

**REGULATION OF TUMOUR GROWTH AND APOPTOSIS  
BY ONCOGENES AND PAPILLOMAVIRUSES**

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## **DECLARATION**

The work reported here was carried out between 1987 and 1991, under the supervision of Dr A. H. Wyllie, in the Department of Pathology, University Medical School, Teviot Place, Edinburgh. The written composition of this thesis is my own work, and the experiments were planned, coordinated and performed by myself, or under my supervision, as stated in the acknowledgements.

## ABBREVIATIONS

AB/PAS	alcian blue/periodic acid Schiff
bp	base pairs
BPV	bovine papillomavirus
CLF	cell loss factor
DDW	deionised distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
E	early viral gene
EDTA	ethylene diamine tetra-acetate
EGTA	ethylene glycol-bis-(B-amino ethyl ether) N, N' tetra-acetate
G418	geneticin
GMEM	Glasgow modification of minimum eagles medium
HPV	human papillomavirus
HINCS	heat-inactivated neonatal calves' serum
HmB	hygromycin B
kb	kilobases
kDa	kilodalton
L	late viral gene
mRNA	messenger RNA
OD	optical density
PBS	phosphate buffered saline
PV	papillomavirus
RNA	ribonucleic acid
rpm	revolutions per minute

SDS	sodium dodecyl sulphate
SSC	standard sodium citrate
SV40	Simian virus 40
TBE	tris borate EDTA
TE	tris EDTA
TGF	transforming growth factor
TPA	12-O-Tetradecanoyl phorbol-13- acetate

Standard quantitative abbreviations (eg L, ml, M, uM, etc), chemical abbreviations (eg ATP,  $\text{Ca}^{2+}$ , etc), and single letter amino acid code have also been used.

## ABSTRACT

The role of HPV genomes in human cervical neoplasia was re-examined using a new, sensitive PCR-based assay. This demonstrated increasing HPV prevalence with increasing grade of histological abnormality in cervical neoplasia. In contrast, HPV was not observed in histologically normal epithelium from a control group of women defined without reference to attendance at clinics for gynaecological or sexually transmitted disease. HPV 16 was associated more with squamous cell carcinomas, and HPV 18 with cancers showing glandular differentiation - the histological type with a poorer prognosis. HPV 18 had higher CANCER/CIN prevalence ratios than HPV 16, indicative of a more rapid transition from CIN to carcinoma. Thus, HPV 18 appeared to be a more aggressive type than HPV 16.

A rodent fibroblast model was developed to investigate the effects of HPVs and the myc and ras oncogenes on tumour cell apoptosis and growth rate. These genes were introduced into immortalised fibroblasts, which were studied both in vitro and in vivo. Regardless of the introduced genes, fibroblast proliferation in vitro differed little between the resulting cell lines. In contrast, the rates of cell death (which was uniformly effected by apoptosis) differed widely. Each tumour cell line had a characteristic rate of apoptosis, which determined large differences between the lines in their

rates of population expansion in vitro. The tumours produced by subcutaneous injection of these cells into immune suppressed mice also showed large differences in size, which demonstrated a consistent relationship to the relative frequencies of mitosis and apoptosis. Thus, apoptosis appeared to be a major determinant of tumour growth in vitro and in vivo. The ratio of apoptosis to mitosis appeared to be characterised by the genes inserted into these cells. The c-myc oncogene and high risk HPV genomes stimulated tumour cell apoptosis. HPV 18 was associated with lower levels of tumour cell apoptosis than HPV 16. In contrast, the activated ras oncogene suppressed apoptosis, either alone or in combination with the other genes, contributing to the faster growth of ras transfected fibroblasts.

The c-myc oncogene was associated with expression by viable cells of one of the effector proteins involved in apoptosis, the calcium-magnesium sensitive endonuclease. In contrast, this enzyme was not expressed by viable cells of the parent fibroblast cell line, or ras transformed fibroblasts. These observations are consistent with the hypothesis that apoptosis occurs in 2 distinct stages: priming - in which new effector molecules appear within the cells; and triggering - in which these molecules are activated to mediate apoptosis. Priming for apoptosis by c-myc may explain the higher intrinsic rates of apoptosis of c-myc transfectants, and perhaps, the apoptotic rates associated with presence of

high risk HPV genomes. Thus, differential genetic regulation of apoptosis is reflected in net tumour growth rates in vitro and in vivo, and may partly explain the more rapid transition from CIN to cancer, and the more aggressive phenotype of cervical cancers containing HPV 18 compared to HPV 16.



## 1. **INTRODUCTION**

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## 1. INTRODUCTION

It is axiomatic that the biological aggressiveness of a tumour determines the prognosis for the patient. Yet, tumour aggression is a poorly defined property, which probably compounds many elements of tumour behaviour. Rather little is known of what the essential elements are, and less of the manner in which they are regulated. The central theme of this thesis is that specific viral and cellular oncogenes regulate two parameters which are of fundamental significance for tumour aggression:

- (1) **progression** of premalignant lesions to malignancy, which is investigated in the human cervix; and
- (2) **growth rate**, which is studied using an experimental fibroblast system.

More specifically, the possible contribution of HPVs to **progression** of CIN to cervical cancer is addressed by measuring the prevalence of viral genomes in these lesions. To do this, without merely reiterating the controversy which has surrounded the association of papillomaviruses with cervical neoplasia, a new, highly sensitive and specific, PCR-based assay was designed.

For practical reasons, it is difficult to study tumour **growth rate** directly in patients. Net tumour growth rate is determined by the balance of cell proliferation and cell loss. Proliferation in tumours - both human and experimental - has been investigated extensively, but the contribution of cell loss to tumour growth and

aggressiveness is less well understood. In the few instances where quantitative data are available tumour cell loss is considerable. This thesis examines the proposition that tumour cell loss by the process of apoptosis plays an important role in determining the growth rate of tumours. Little is known about the genes that regulate tumour cell apoptosis. Candidate genes include those involved in modulating other aspects of cell regulation, including proliferation and differentiation, such as cellular and viral oncogenes. The myc and ras oncogenes and the human papillomavirus genomes were selected because of their known involvement in human tumours. Their effects on tumour cell gain and loss were investigated by manipulating their expression in immortalized rodent fibroblasts.

To introduce this work, this chapter reviews four subjects: tumour aggression and growth, apoptosis, myc and ras oncogenes, and human papillomaviruses.

## 1.1 TUMOUR AGGRESSION AND GROWTH

Human tumours derive from single cells and are thought to evolve into malignant tumours by a series of stepwise genetic changes (Nowell, 1976; Bernstein and Weinberg, 1985). Tumour cell variants develop, perhaps as a manifestation of genetic instability, within the expanding tumour cell population. Many of these variants are probably eliminated because of growth disadvantage, loss of key metabolic functions, immunological destruction, or genetically determined cell loss. However, occasionally variant cells may arise which have a selective advantage over their parent cells, and expand to form a new tumour subclone (Nowell, 1976). Many malignant tumours studied have been found to be heterogeneous, reflecting multiple subclones, in many aspects of genotype and phenotype, including morphology, ploidy, and proliferation (Shapiro et al, 1981; Fidler and Hart, 1982; Moore, 1983; Carey et al, 1990; Hall et al, 1990).

The probability of generating variant subclones with greater aggression, depends on the overall rate of population expansion of viable tumour cells, which is determined by the balance of cell gain and loss. Furthermore, the mechanism of cell loss may play a role in the selection of aggressive subclones for survival or deletion.

### 1.1.1 TUMOUR AGGRESSION

The concept that some tumours are more aggressive than others is intuitively straightforward and clinically important, yet tumour aggression is poorly understood and correspondingly difficult to measure. In the absence of more precise indices, clinico-pathological measurements are often used, including death rates relative to the tumour incidence rates, recurrence rates after therapy, and survival rates at various times after diagnosis (often 5 years). Histopathological measurements of tumour aggression include tumour cell type, grading and staging (Cotran et al, 1989). These indices can obscure as well as reveal differences in tumour aggression. A tumour with large primary size and lymph node metastases may have been present for only a short time prior to diagnosis, in which case it is obviously aggressive; or it may be much less aggressive, having achieved this status after more than a decade of slow growth, all unnoticed by the patient or physician. Underlying these differences are poorly defined biological properties that determine the tumour's capacity for expansion, infiltration and metastasis (reviewed by Sobel, 1990).

Cell surface receptors are required for attachment of tumour cells to basement membranes or the extracellular matrix, such as the laminin receptor (Liotta et al, 1988). Whereas, absence of other surface molecules (eg E-cadherin) apparently promotes invasion (Birchmeier et al,

1991). Secretion of degradative enzymes may clear a path for invasion, for example collagenase type IV specifically cleaves collagen type IV in basement membranes (Liotta et al, 1988). Induction of stromelysin 3 in stromal cells by tumour, or induction of abnormal stromal matrix components (eg tenascin) may generate new spaces in basement membranes and tissue stroma. Autocrine motility factors (eg AMF or scatter factor) are produced, which promote movement of tumour cells into these spaces. Some of these have been found to be associated with activation of certain oncogenes, for example collagenase type IV secretion and ras expression (Liotta et al, 1988). Still other factors (eg nm23, CD44) relate to poor prognosis or to the frequency of metastasis, but for obscure reasons (Birchmeier et al, 1991). It is clear that the biology of tumour aggression is understood only imperfectly at present.

Whatever else is involved, tumours behave as they do because they contain cells, with properties essential for the neoplastic phenotype. The generation of such cells, including those with new properties, must therefore be important. Autonomous cell proliferation is the fundamental phenotypic change in neoplasia, and reflects escape from normal growth control. The mechanism and degree of growth deregulation is likely to influence the net rate of growth of the tumour cell population.

### 1.1.2 TUMOUR CELL PROLIFERATION

The molecular basis of autonomous tumour cell proliferation is linked to two major groups of genes - oncogenes, including those encoding growth factors, and tumour suppressor genes. Mitogenic signalling pathways mediate the normal functions of growth factors, and oncogenes function at critical steps in these pathways. Oncogenes encode growth factors (eg sis), growth factor receptors (eg erb-B1), post-receptor signalling molecules such as tyrosine kinases (eg src) and G proteins (eg ras), and nuclear factors (eg myc). Activation of oncogenes, either by mutation or increased expression through a variety of means, results in subversion of signalling pathways in cancers (reviewed by Bishop, 1991).

Growth factors are involved in entry into the cell cycle, from G0 to G1 - "competence factors" (eg EGF, PDGF, or FGF), and progression from G1 to S phase - "progression factors" (eg IGF-1, or insulin). Oncogenes appear to replace the actions of the former in tumours, but the requirement for the latter may explain growth factor dependence of many established cancer cell lines (reviewed by Aaronson, 1991). Growth factors also influence tumour cell differentiation and non-tumour host cells, such as those of the stroma and vasculature, affecting overall tumour growth.

Tumour suppressor genes may act as a growth-restraining

counterbalance to growth-promoting oncogenes, and inactivation of them appears to liberate the cell from such constraints. Many tumour suppressor genes were identified by observation of loss of heterozygosity (eg in retinoblastoma, or familial adenomatous polyposis coli), and inherited mutation is associated with congenital predisposition to specific cancers (reviewed by Weinberg, 1991). Their mechanisms of action are not fully understood, although polypeptide homologies suggest some similarities, for example between DCC and cell adhesion molecules, and also between NF-1 and GTPase activating proteins. Understanding of the regulatory circuitry that governs tumour cell proliferation is incomplete, but the early evidence points to important interactions between growth factors, oncogenes and tumour suppressors.

Cell proliferation has been studied extensively in many tumour types and has often proved to be a very inaccurate predictor of local growth rate (Quinn and Wright, 1990). This is presumably because net population expansion rate is a balance of cell proliferation and cell loss. It is the central hypothesis of this thesis that this balance is significant in determining tumour aggression, and is influenced by genes that regulate not only cell proliferation, but also cell loss.



### 1.1.3 TUMOUR CELL LOSS

Cells may be lost from tumour cell populations by a variety of mechanisms. Cells may depart the primary tumour site by natural exfoliation or cell migration. They may leave the proliferating pool of cells by terminal differentiation, or they may die (Cotran et al, 1989). Previous descriptions of tumour cell death have often focussed on necrosis (Thomlinson and Gray, 1955; Steel, 1977; Moore, 1983 and 1987; Tozer et al, 1990), with less extensive study of apoptosis in tumours (Kerr et al, 1972; Wyllie, 1985). Quantitative estimates of net tumour cell loss have been made (Steel, 1977; Moore, 1983 and 1987).

It is possible to calculate the potential doubling time ( $T_p$ ) from estimates of tumour cell production rates (mitotic indices, nucleotide incorporation or fraction of cycling cells), and the actual doubling time ( $T_d$ ) from measurements of tumour volume, and so derive the cell loss factor ( $CLF = 1 - T_p/T_d$ ). The CLF approaches zero with no cell loss, and unity if extensive loss makes the actual doubling time unmeasurably large. In deriving CLFs, errors can arise from several inevitable assumptions, but in almost every tumour studied the CLF is large. In rodent sarcomas and carcinomas CLFs of 0.65-0.78, and in human bronchial and colorectal carcinomas and a malignant melanoma CLFs of 0.73-0.96 were recorded (Steel, 1977; Moore, 1983 and 1987; Kerr and Lamb, 1984).

The high level of tumour CLFs presumably explains the poor correlation between measures of cell proliferation, such as the thymidine labelling index, and the tumour volume doubling times for a whole range of human tumours (Steel, 1977) and points to the considerable importance of tumour cell loss in determining tumour growth rate. Thus, when the cell loss factor is large, it is likely to act as a major regulator of the rate of tumour expansion and probably determines the overall growth pattern in terms of tumour enlargement, stasis or regression.

There is an unresolved question of whether the cell loss factor is an intrinsic characteristic of a tumour cell line, or is simply a manifestation of the growth environment, *in vivo* or *in vitro*. The CLF has also been shown to vary during enlargement of tumours over time (Lala, 1972; Steel, 1977; Sarraf and Bowen, 1986). Variations in the CLF with time may perhaps be explained by elevated levels of cell death by necrosis, when tumour cell populations outgrow their blood supply, or by increased immune attack of tumour cells.

It is clear that cell loss occurs in tumours and that it is relevant to the kinetics of tumour cell population expansion, but there is surprisingly little direct quantitative data on the relative contributions by different mechanisms of cell migration, exfoliation, differentiation, and death. It has been argued that cell death is numerically the most significant form of cell

loss from a growing tumour (Kerr et al, 1972; Wyllie, 1985; Bowen and Bowen, 1990). This may occur either by necrosis or apoptosis. Although necrosis is often more conspicuous histopathologically, due to its occurrence in large, confluent zones, it does not occur in sufficient quantities to explain the high tumour cell loss factors observed, in contrast to the comparatively rapid and inconspicuous cell death by apoptosis that occurs in a scattered distribution (Wyllie, 1985; Bowen and Bowen, 1990). The ensuing sections of this chapter therefore review the role of apoptosis in neoplasia, including its incidence, morphology, mechanisms, and differentiation from necrosis. All of these have a bearing on how and why apoptosis may be triggered in growing tumours and its quantitation both in vivo and in vitro.

## 1.2 APOPTOSIS

Cell death plays a major role in the organisation of the cell societies which we call tissues. Sometimes it is conspicuous and clearly pathological, such as when sheets of cells die in synchrony by necrosis (eg infarction). Structurally, necrotic cells show critically damaged organelles (eg mitochondria with 'high amplitude swelling'), ruptured plasma membranes, and dispersal of cytoplasmic elements into the extracellular space (reviewed by Trump et al, 1981; Wyllie, 1981). The mechanisms are various, but do not depend upon continuing synthetic activity. There is no evidence that specific signalling pathways are involved. There is breakdown of membrane homeostasis and net flow of water into the necrotic cell, whose density falls. Intracellular calcium rises uncontrollably to equilibrate with the millimolar concentrations in the extracellular space. The process results in an acute inflammatory reaction, perhaps triggered by complement-activating factors emanating from mitochondria which have escaped from the damaged cell (Kagiyama et al, 1989). Alternatively, leukotrienes and other arachidonate chemotaxins may be generated from partially degraded cell membranes (Denzlinger et al, 1985). The arrival of neutrophil polymorphs permits digestion and phagocytosis of the constituents of the necrotic cells, but brings with it the risk of further tissue damage (Romson et al, 1983). In all these features (incorporating incidence, morphology and mechanism), the

cell biology of necrosis contrasts strongly with that of apoptosis.

#### 1.2.1 INCIDENCE OF APOPTOSIS

Apoptosis occurs frequently (though not exclusively) in circumstances to which the term "programmed cell death" has been applied (table 1.1) (reviewed by Kerr et al, 1972; Wyllie et al, 1980; Wyllie, 1987a; Arends and Wyllie, 1991). These include physiological states, such as atrophy and normal embryonic development (Walker, 1987; Handyside et al, 1986), and pathological states, for example inflammation and tumour growth. Amongst these are the **ontogeny and effector mechanisms of the immune system**. Apoptosis occurs in deletion of autoreactive T-cell clones during thymic maturation, or following stimulation of thymocytes by glucocorticoids or ligand binding of the TCR/CD3 complex (Wyllie and Morris, 1982; Smith et al, 1989; Shi et al, 1989 and 1990). It occurs in B cell deletion in germinal centres in the absence of antigen driven positive selection of centrocytes (Liu et al, 1989). Apoptosis is also found in cells attacked by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Sanderson, 1981; Russell, 1983; Duvall and Wyllie, 1986; Martz and Howell, 1989; Arends and Wyllie, 1991).

Apoptosis plays a critical role during **resolution of acute inflammation**. Neutrophil polymorphs at the end of

their physiological lifespan undergo apoptosis, with subsequent phagocytosis by macrophages. This disposes of them without causing further amplification of the inflammatory response, which would occur if the neutrophils died by necrosis, releasing their granule contents into the tissues (Savill et al, 1989a and 1990).

Apoptosis occurs in **growing and regressing tumours** (Kerr et al, 1972; Moore 1983 and 1987; Wyllie 1985; Sarraf and Bowen, 1986; Bowen et al, 1988; Bowen and Bowen, 1991). It has often been observed as the mode of tumour cell death following cytotoxic therapy (Searle et al, 1975) and recently has been induced in experimental B and T leukemic cells by treatment with monoclonal antibodies to a specific surface epitope (APO-1) (Trauth et al, 1989; Debatin et al, 1990). APO-1 has been cloned, as has fas another cell surface receptor capable of inducing apoptosis, and these differ by only one non-essential amino acid and share homology with the EGF receptor (Itoh et al, 1991). In all these circumstances apoptosis is delineated by a series of strikingly similar morphological changes.

TABLE 1.1

## INCIDENCE OF APOPTOSIS

CIRCUMSTANCE	EXAMPLE
Normal embryonic/foetal development	Blastocyst inner cell mass modelling Palatal fusion Interdigital web deletion Avian wing development-"posterior necrotic zone" Mullerian duct regression in the presence of foetal testis Supernumerary motor neuron deletion in the absence of end-plate formation Intestinal villo-cryptal modelling Adrenal zona reticularis Amphibian tail regression Caterpillar labial feeding gland regression Pancreatic duct obstruction Endometrium: cyclic proliferation and deletion Breast: cyclic proliferation and deletion Ovary: corpus luteum regression Adrenal: ACTH withdrawal Prostate: castration IL-2 withdrawal from thymocytes IL-3 withdrawal from haemopoietic precursors Serum withdrawal from fibroblasts Atrophy of lead nitrate-induced rat liver hyperplastic foci Restitution of hypercellular glomeruli Autoreactive T cell clone deletion B cell deletion in the absence of antigen-driven centroyte selection
Growth factor dependent survival	Cytotoxic T cell killing Natural killer cell killing K cell killing
Involution of hyperplasia	Lymphotoxin-mediated killing Gliotoxin-induced macrophage death TCD-dioxin-induced thymocyte death DMBA-induced adrenocortical cell death Polycyclic hydrocarbon-induced teratogenesis Thymic cortical cell death Intestinal mucosal crypt cell death
Immune cell ontogeny	Neonatal renal cortical nephrogenic cell death Disposal of unstimulated senescent neutrophil polymorphs Basal and squamous cell skin carcinomas Antibody-induced leukaemic cell death Regression of endocrine-dependent tumours Treatment with cancer chemotherapeutic agents
Immune killing	
Toxin exposure	
Teratogenesis	
Irradiation	
Resolution of inflammation	
Tumour cell deletion	

### 1.2.2 MORPHOLOGY OF APOPTOSIS

The morphological changes of apoptosis occur in three phases (Kerr et al, 1972; Wyllie et al, 1980; Wyllie, 1987a and 1988; Walker et al, 1988b; Arends et al, 1990; Arends and Wyllie, 1991). In the first, there is reduction in nuclear size, condensation of chromatin into toroids or crescentic caps at the nuclear periphery and nucleolar disintegration with dissociation of the transcriptional complexes from the fibrillar centre. Cells dying by apoptosis detach themselves from their neighbours and from culture substrata. There is loss of specialised surface structures, such as microvilli and contact regions. The cell adopts a smooth contour. Cell volume shrinks, cytoplasmic organelles become compacted, and the smooth endoplasmic reticulum dilates. The dilated cisternae fuse with the cell membrane, giving rise to a bubbling appearance at the surface. Cytoskeletal filaments aggregate in side-to-side arrays, often parallel to the cell surface, and ribosomal particles clump in semi-crystalline formations, but otherwise the organelles remain intact.

In contrast to necrosis, the other major type of cell death (Trump et al, 1981), mitochondria do not show "high amplitude swelling", the cell membrane does not become permeable to vital dyes at this stage, and apoptotic cells within tissues do not elicit an acute inflammatory reaction (Wyllie, 1981).



In phase 2 (which may overlap with the first), there is blebbing at the cell surface and crenation of the nuclear outline. Both nucleus and cytoplasm may split into fragments of various sizes. Typically, the cell becomes a cluster of round, smooth, membrane-bounded "apoptotic bodies", some containing nuclear fragments, others without. These bodies may be shed from epithelial surfaces or phagocytosed by neighbouring cells or macrophages. In glandular tissues in particular, intraepithelial macrophages are prominent in this activity (Walker, 1987; Walker and Gobe, 1987).

In phase 3, there is progressive degeneration of residual nuclear and cytoplasmic structures. In cultured cells, this is manifested as membrane rupture producing permeability to vital dyes. In tissues these changes (sometimes termed "secondary necrosis") usually occur within the phagosome of the ingesting cell. Eventually membranes disappear, organelles become unrecognisable and the appearance is that of a lysosomal residual body. The majority of apoptotic bodies seen in tissues studied with the light microscope are in this phase, and sometimes the smooth outline of the ingesting phagosome can be seen around them, but earlier phases can also be recognised by their rounded contours and deeply hyperchromatic, often fragmented nuclei. The histological appearance familiar to pathologists as "melanosis coli" is the accumulation in the lamina

propria of macrophages laden with apoptotic bodies derived from mucosal cells (Walker et al, 1988a). "Tingible body macrophages" in lymph node reactive centres are similar, being laden with the apoptotic residues of lymphocytes.

Time lapse cinematographic studies of apoptosis reveal the sudden onset of cell shrinkage, with surface blebbing and bubbling, as cells enter phase 1 and 2, after a variable time from exposure to the lethal stimulus (Sanderson, 1981; Russell, 1983). This initial response lasts for only a few minutes, and generates small dense apoptotic cells. If not phagocytosed immediately these cellular particles undergo a gradual loss of cell density (Wyllie, 1985), coinciding with loss of membrane integrity, shown ultrastructurally and by failure to exclude vital dyes. Apoptotic cells remain recognisable within tissues for 4-9 hours, a time-course which coincides with that of complete degradation of other large biological structures within the phagosomes of macrophages. This relatively short period ensures that high rates of apoptosis produce only small increases in the proportion of apoptotic cells observed in tissue sections. Very simple calculations show, for example, that if a tissue were to undergo involution to half its cell number over 3 days, by a steady rate of apoptosis, each apoptotic body remaining recognisable for 6 h, then the proportion of apoptotic cells evident on microscopy would rise to just over 4% in the first 6h, and

thereafter more slowly to just over 8% by the end of the 3rd day.

### 1.2.3 MECHANISMS OF APOPTOSIS

The biochemistry of apoptosis is less well defined than its morphology, probably because this process characteristically involves scattered single cells within tissues, surrounded and outnumbered by viable neighbours. Nonetheless, six major events are known.

#### 1.2.3.1 CELL DENSITY INCREASE

The conspicuous volume reduction of apoptotic cells initially led to this type of death being called "shrinkage necrosis" (Kerr, 1971; Kerr et al, 1972). In thymocytes this is associated with a pronounced single step-wise increase in buoyant density, suggesting that a proportion of intracellular water and ions are lost, without corresponding loss of macromolecules or organelles (Ohyama et al, 1981; Wyllie and Morris, 1982). In apoptotic cells endoplasmic reticulum (ER) dilates forming vesicles which fuse with the plasma membrane, voiding their contents extracellularly (Morris et al, 1984). This rapid and selective export of fluid and intracellular ions into the ER may be mediated by an ionic transporter system. A sodium-potassium-chloride cotransporter has been described, inhibition of which leads to net loss of sodium and water from affected cells

(Wilcock et al, 1988). At this time, apoptotic cells show no evidence of increased permeability to vital dyes or increased loss of previously accumulated radioactive chromium. At a more pragmatic level this density increase allows purification of intact apoptotic cells by density centrifugation for experimental purposes (Wyllie and Morris, 1982).

#### 1.2.3.2 INTRACELLULAR SIGNALLING PATHWAYS

Perception of physiological lethal stimuli is presumably mediated by cell type- and stimulus-specific receptors. Possible intracellular signalling mechanisms in the initiation of apoptosis include ion fluxes, phosphoinositide hydrolysis, changes in activity of protein kinases and altered expression or activity of oncogenes. Although there is shut-down of total protein and RNA synthesis early in apoptosis (Wyllie and Morris, 1982) in some cell types initiation of the process appears dependent upon protein synthesis and can be abrogated by application of inhibitors such as cycloheximide or actinomycin D, shortly after the lethal stimulus (Wyllie et al, 1984b; Wyllie, 1985).

An interesting example of cell-type and stage-specific signal transduction is the response of T cells to CD3 ligand binding: whereas in post-thymic cells the response is proliferation, in the immature intrathymic cells it is apoptosis (Smith et al, 1989; McConkey et al, 1989b).

Similar observations have been made in T cell clones, where apoptosis is blocked by cyclosporin A and inhibitors of protein and RNA synthesis (Shi et al, 1989). As in the apoptosis induced by glucocorticoid, the response to CD3 binding is preceded by a sustained rise in cytosolic  $\text{Ca}^{2+}$  (McConkey et al, 1989a and 1989b). Apoptosis in thymocytes can also be induced using low doses of calcium ionophore (Wyllie et al, 1984b). Endonuclease activation (in intact cells or even in isolated thymocyte nuclei incubated in  $\text{Ca}^{2+}$ ) is inhibited by phorbol ester (TPA), an agent which stimulates endogenous protein kinase C (PKC), and this can be reversed by H-7, a supposedly specific PKC inhibitor (McConkey et al, 1989b and 1989c). Thus calcium mobilisation without commensurate stimulation of PKC may trigger apoptosis in suitably primed cells. Initial reports of direct measurements of diacyl glycerol and phosphoinositides in apoptotic thymocytes suggests that the different responses of intrathymic and post-thymic cells are not due to differences in these signalling pathways (Conroy et al, 1991).

In prostatic epithelium, castration initiates a cascade of transcriptional activation involving c-fos, c-myc and hsp-70 prior to the onset of apoptosis (Buttayan et al, 1988). Novel RNA transcripts and proteins have been observed following apoptosis of prostatic epithelium and thymocytes (Montpetit et al, 1986; Colbert and Young, 1986). Two such transcripts in thymocytes (RP-2 and RP-8)

have been partially characterized: one has a transmembrane sequence suggestive of a cell surface protein, and the other has a zinc finger motif suggestive of a DNA regulatory protein (Owens et al, 1991). Transcriptional activation of the TRPM-2 gene, which has homology with sulphated glycoprotein-2 gene (SGP-2 or clusterin) expressed in sertoli and epididymal cells, has been demonstrated following the onset of apoptosis in prostatic epithelium after castration and also in breast epithelium (Kyprianou et al, 1990). Withdrawal of certain specific growth factors or transducers (eg IL-2, or bcl-2 protein) from lymphoid cell lines in culture initiates apoptosis, although the factors themselves do not necessarily stimulate proliferation (Duke and Cohen, 1986; Vaux et al, 1988). Apoptosis of rat chloroleukaemic cells was preceded by repression of c-Ki-ras expression (Servomaa and Rytomaa, 1988). Wild type p53 activity induced apoptosis in a myeloid leukaemic cell line, whereas switching the temperature to effect the mutant p53 activity of the temperature-sensitive p53 protein did not do so (Yonish-Rouach et al, 1991). It appears likely that the oncogene and growth factor-dependent signal transduction pathways, for long interpreted rather single-mindedly in terms of cell proliferation, will be shown to play additional important roles in the regulation of cell survival and death.

The genetic regulation of programmed cell death has been

well characterised in the nematode *Caenorhabditis elegans*. Analysis of mutations yielding abnormal cell death phenotypes has identified three loci in particular: *ced-3*, *ced-4* and *ced-9*. Recessive mutations at the *ced-3* and *ced-4* loci block nearly all of the programmed cell deaths that occur during *C. elegans* development (Ellis et al, 1991). It has been suggested that these genes code for specific functions required in the dying cell itself (Yuan and Horvitz, 1990). Some mutations of the *ced-9* gene with recessive transmissibility exhibit an embryonic lethal phenotype, consistent with constitutively activated cell death. Other types of mutation of *ced-9* exhibiting dominant transmissibility are phenotypically similar to *ced-3* and *ced-4* mutations. These observations suggest that the *ced-9* product may act as a type of master-switch able to positively or negatively regulate the cell death pathway (Ellis et al, 1991). These incisive studies promise to shed much light on the genes regulating death in general, including mammalian apoptosis. Despite the species divergence many of the structural features of programmed cell death in the nematode are similar to those of apoptosis. So far, however, no homologues to *ced-3*, *ced-4*, or *ced-9* have been identified in mammals.



### 1.2.3.3 CHROMATIN CLEAVAGE

Internucleosomal chromatin cleavage is associated almost exclusively with the morphology of apoptosis. This association was first demonstrated in glucocorticoid-treated rat thymocytes in 1980 (Wyllie). Cleavage of internucleosomal linker DNA generates well organised chains of oligonucleosomes, with DNA lengths which are integer multiples of 180-200 bp - the size of DNA wrapped around a single histone octamer - observed as a ladder on gel electrophoresis (Hewish and Burgoyne, 1973). The typical "chromatin ladder" has now been reported along with morphological chromatin condensation of apoptosis in many cell systems (Rotello et al, 1989; Zeleznik et al, 1989; Vaux et al, 1988; Baxter et al, 1989). The only known circumstances in which endogenous chromatin cleavage is not accompanied by the complete morphology of apoptosis are normoblast maturation and the differentiation of lens epithelium (reviewed in Wyllie, 1987a). In both of these, although some of the cytoplasmic changes are atypical, nuclear chromatin undergoes widespread condensation entirely similar to that of apoptosis. In contrast, cell death by necrosis is not associated with internucleosomal DNA cleavage (Russell, 1983).

It has recently been demonstrated that DNA cleavage in apoptosis occurs selectively, without associated chromatin proteolysis (Arends et al, 1990a). The nuclear



matrix appears normal, in terms of structural organisation and the presence of the most abundant protein species. DNA cleavage is at widely dispersed sites: the apoptotic nucleus has a normal content of acid-precipitable DNA. Two classes of chromatin fragments are generated (Arends et al, 1990a): 70% exists as oligonucleosome fragments bound to the nucleus, whilst 30% is unattached. Although the bound chromatin includes fragments as short as dinucleosomes, the majority are long; in contrast, the free chromatin comprises mono- and short oligonucleosome fragments only. This minority class probably derives from chromatin in a transcriptionally active configuration as the chromatin-bound proteins are depleted of histone H1 and enriched in high mobility group (HMG) proteins 1 and 2 - changes associated with active gene transcription (Tremethick and Malloy, 1988). Whereas inactive heterochromatin is thought to be tightly wound in a solenoid (Finch and Klug, 1976), transcriptionally active chromatin is not compacted in this way, which would allow better access to enzymes in the nucleoplasm, producing more complete digestion. The pattern of chromatin digestion in apoptosis, therefore, is consistent with activation of an endonuclease in solution in the nucleoplasm, rather than a constituent of the matrix itself.

Brief digestion of normal nuclei with a purified exogenous endonuclease (micrococcal nuclease) in the presence of protease inhibitors, reproduces the nuclear

morphologic changes of apoptosis, in step with generation of the typical DNA ladder (Arends et al, 1990a). Interestingly, these changes include those observed in the apoptotic nucleolus: segregation and dispersal of the dense fibrillar and granular components with preservation of an intact fibrillar centre. These may be explained in terms of cleavage of the transcriptionally active ribosomal genes within the dense fibrillar component, with conservation of the inactive ribosomal DNA protected within the nucleolin-rich fibrillar centre.

Thus, there is good evidence that the characteristic morphological condensation of chromatin in apoptosis is due to DNA cleavage. It is more difficult to be certain that DNA change in apoptosis is due to an endogenous endonuclease. It has been suggested that in some circumstances similar chromatin cleavage might be the result of damage by reactive oxygen intermediates (Balkwill et al, 1989). Three observations, however, make this improbable. First, cells dying by necrosis (in which there is at least as much precedent for generation of reactive oxygen intermediates as in apoptosis) do not show the characteristic "chromatin ladder" (Russell, 1983). Second, the DNA cleavage in apoptosis is predominantly double-stranded, with no single-stranded nicks or gaps detectable by incubation with S1 nuclease (Arends et al, 1990a), whereas free radical damage would be expected to generate a high proportion of single

strand breaks. Third, in thymocytes subjected to ionizing radiation, where free radicals are known to mediate many biological effects, the evolution of apoptosis shows a different timescale from the ionization events which generate free radicals (reviewed in Wyllie, 1985). Within seconds of radiation exposure, cellular DNA undergoes multiple single-strand breaks, with consequent relaxation of supercoiling, characteristic of ionization damage (Filippovich et al, 1982). These breaks are repaired within minutes, and at this stage there is no apoptosis. About an hour later, however, the cells begin to show morphological apoptosis, together with internucleosomal double-strand chromatin cleavage, processes which can be abrogated by treatment (after the radiation) with inhibitors of protein synthesis (Yamada and Ohyama, 1988).

Early experiments with thymocyte nuclei suggested that they contained an enzyme capable of cleaving chromatin in apoptosis. If incubated in neutral pH, together with both calcium and magnesium, such nuclei quickly developed multiple double-strand DNA breaks, generating the familiar "ladder" on electrophoresis (Duke et al, 1983; Cohen and Duke 1984). This activity can be inhibited by zinc ions (Flieger et al, 1989). Endonucleases with suitable features are known to be present within the nuclei of many cell types (Hewish and Burgoyne, 1973; Ishida et al, 1974; Nakamura et al, 1981; Liu et al, 1980) and it seemed plausible that the raised calcium

levels within apoptotic cells might be sufficient to activate this enzyme. Attempts to purify an enzyme with properties of the thymocyte neutral nuclease have been reported (Wyllie et al, 1986b; Dykes et al, 1987).

The thymocyte nuclease cleaves chromatin of a target system (nuclei from cells labelled during growth with tritiated thymidine) to release labelled oligonucleosomes. This nuclease activity is optimum at pH 7.5, in contrast to contaminating acid nucleases, which also differ in cleaving DNA to much smaller (acid-soluble) fragments. The neutral calcium-magnesium endonuclease is maximally eluted from normal thymocyte nuclei at 300mM NaCl, and appears to be an anionic protein of molecular weight around 130kd (Wyllie, unpublished observations). This is substantially larger than other candidates reported previously (Compton and Cidlowsky, 1987) and now disputed (Alnemri and Litwack, 1989), but is close to the size of one subunit of topoisomerase II (Halligan et al, 1985). Topoisomerase II is known to be present in thymocytes and would be capable of engendering double-stranded DNA cleavage under appropriate conditions (eg low ATP) (Udvardy et al, 1986; Chow and Ross, 1987). It is not clear, however, whether the thymocyte Ca-Mg endonuclease is identical with topoisomerase II, or indeed whether either is responsible for the chromatin changes in apoptosis.

In thymocytes the Ca-Mg endonuclease is constitutively

present (Alnemri and Litwack, 1989). Whereas it is absent from certain human and murine lymphoid cell lines (Alnemri and Litwack, 1990). When cells of these lines underwent apoptosis in vitro in response to glucocorticoid, however, the extractable Ca-Mg endonuclease activity rose from low levels, peaking as endogenous chromatin cleavage and the morphology of apoptosis appeared (Wyllie et al, 1986a). None of these changes occurred in sublines selected for glucocorticoid resistance. Similar nuclease activity has been observed in an entirely different cell system, the physiological death of ovarian corpus luteum cells (Zelevnik et al, 1989). Thus, endogenous endonucleases remain interesting candidates amongst the effectors of apoptosis, but their induction can precede the event of apoptosis itself.

#### 1.2.3.4 TRANSGLUTAMINASE ACTIVATION

Coincident with the onset of apoptosis during the involution of liver hyperplasia and in glucocorticoid-treated thymocytes, there is induction and activation of tissue transglutaminase (Ca<sup>2+</sup>-dependent protein-glutamine  $\gamma$ -glutamyltransferase) (Fesus et al, 1987). Transglutaminases cross-link proteins through  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds and mediate both formation of cornified envelopes by epidermal keratinocytes (Green, 1980), and cross-linking of fibrin and 2-plasmin inhibitor in the final stages of thrombus stabilisation (Sakata and Aoki, 1982). In apoptosis there is an

increase in transglutaminase mRNA and protein, enzyme activity and protein-bound ( $\gamma$ -glutamyl) lysine (Fesus et al, 1987; Fesus et al, 1991). The probable consequence of transglutaminase activation is an extensive cross-linking of cytoplasmic and membrane proteins. In fact apoptotic cells contain protein shells insoluble in detergents and chaotropic agents. These shells, which are not extractable from normal cells, appear in scanning EM as wrinkled, spherical structures with some morphologic similarities to epidermal cornified envelopes (Fesus et al, 1989). This action may limit escape of potentially toxic intracellular contents (eg lysosomal enzymes) that may otherwise excite an inflammatory response (Fesus et al, 1991).

#### 1.2.3.5 CELL SURFACE ALTERATIONS

It is characteristic of apoptotic cells that they are rapidly recognized and phagocytosed by their neighbours or by macrophages. The recognition process has been reproduced in vitro in two isologous systems. Macrophages bound preferentially to apoptotic compared with normal cells. Recognition of apoptotic mouse thymocytes was mediated by a sugar-dependent mechanism, inhibited by N-acetylglucosamine or its dimer, N, N'-diacetylchitobiose, but not by mannose or fucose, and only to a slight extent by other monosaccharides including galactose (Duvall et al, 1985).

A similar sugar-dependent binding was demonstrated in the recognition of apoptotic, aging human neutrophils by isogeneic macrophages, although here glucosamine, galactosamine and mannosamine inhibited recognition, as did the basic amino acids L-lysine and L-arginine, and the inhibition reactions were pH-sensitive and localized to the apoptotic neutrophil surface (Savill et al, 1989b). This sugar inhibition pattern suggests a lectin-type interaction of the apoptotic thymocyte with a receptor-like molecular complex on the surface of rodent macrophages capable of recognizing exposed glycan groups on the surface of apoptotic cells. GlcNAc, the sugar recognized in rodent thymocyte apoptosis, is present only in deep positions within glycan structures of mature glycoproteins and glycolipids. Other sugars, including galactose and charged sialyl groups, are added superficially during processing in the golgi apparatus (Kornfield and Kornfield, 1985). There is independent evidence that these superficial groups may be lost in apoptosis, from the observed reduction in cell surface charge density, measured by microelectrophoretic mobility (Morris et al, 1984).

Apoptotic cell surfaces lose existing cell membrane due to surface blebbing and budding, with shedding of microvilli, and gain new membrane through fusion of vesicles of dilated ER (Wyllie et al, 1980; Wyllie, 1987a). Although other mechanisms are possible including the expression of specific cell surface receptors (Wyllie



et al, 1984a), this membrane loss and replacement could explain the change from the normal population of mature surface glycan groups to one containing some immature glycan groups, leading to focal exposure of sugars normally found in the interior of glycan structures, such as GlcNAc (Morris et al, 1984; Wyllie 1987a). Macrophages and hepatocytes are also known to clear blood glycoproteins (gp) which have lost terminal sugar residues, either asialo-gp (galactose terminated) or asialoagalacto-gp (GlcNAc terminated). In each case the clearance was found to be mediated by specific cell receptors on the phagocytic cells (Ashwell and Hartford, 1982; Drickamer, 1988).

Macrophage recognition and phagocytosis of apoptotic human neutrophils and lymphocytes can also be inhibited by the RGDS tetrapeptide (Arg-Gly-Asp-Ser), RGD bearing proteins vitronectin and fibronectin, or monoclonal antibodies specific for the vitronectin receptor polypeptide subunits (Savill et al, 1990). The inhibitory effect was localized to the macrophage cell surface, from which the vitronectin receptor polypeptide subunits were immunoprecipitated, demonstrating that recognition of apoptotic cells involves the vitronectin receptor (Savill et al, 1990), a member of the  $\beta 3$  cytoadhesin family of integrins (Hynes, 1987).

Thus, it is likely that recognition of apoptotic cells by macrophages or neighbouring cells involves existing



specific receptors on acceptor cells binding to newly-exposed ligands on apoptotic cells by integrin-peptide and lectin-carbohydrate type interactions. It is not known whether there is a direct interaction between acceptor cell receptors and apoptotic cell ligands, or whether adherence is mediated via a molecular bridge, such as occurs during platelet aggregation in which fibrinogen bridges GPIIbIIIa integrins on platelet surfaces (Phillips et al, 1988). Molecular bridging would be compatible with the inhibition data, as lectin-carbohydrate interactions may occur between one end of the bridge and the apoptotic cell surface, and integrin-peptide interactions between the other end of the bridge and the macrophage cell surface (Savill, 1990). It is also not known whether single or multiple receptor-ligand or receptor-bridge-ligand interactions are required for recognition and adherence. Diverse mechanisms might be expected, however, as apoptotic cells can be phagocytosed by neighbouring parenchymal cells as well as macrophages. There are precedents for multiple, and sometimes synergistic, receptor recognition mechanisms in other cell-cell interactions mediated by integrins (Hynes, 1987; Dransfield et al, 1990).

#### 1.2.4 A GENETIC PROGRAMME FOR APOPTOSIS

A coordinated but complex pattern of events, such as those seen morphologically and biochemically in apoptosis, indicates the existence of a genetic programme

for cell death. This implies genetic regulation of the programme, in order that it may be switched on or off appropriately in time and space. However, little is known of such putative controller genes. The role of apoptosis in neoplasia and how it may be regulated by candidate genes - in particular the ras and myc oncogenes and the transforming genes of papillomaviruses - is the central theme of this thesis.

### 1.3 MYC AND RAS ONCOGENES

#### 1.3.1 MYC ONCOGENES

##### 1.3.1.1 MYC GENE STRUCTURE AND FAMILY

The c-myc oncogene was first identified as a cellular homologue of the transforming sequence in the MC29 avian retrovirus (reviewed by Varmus, 1984). However, only the second and third exons of avian, mouse and human cellular c-myc are homologous to the MC29 v-myc sequence, and these contain the protein encoding sequences. The first exon consists of a 400-500 bp untranslated segment, without initiation codons and with stop codons in all reading frames (Stanton et al, 1983), which is involved in regulation of c-myc expression (Cole, 1986). There are two small domains of 70 and 80 bp ("myc boxes"), separated by 120-150 bp in the middle of the c-myc second exon, which showed a high degree of homology to non-c-myc sequences, which were used to identify other genes in the myc family. Five related proto-oncogenes have been found in this myc gene family: c-myc, N-myc (found to be amplified in double minute and homogenously staining chromosomal regions in neuroblastomas), L-myc (similar amplification in variant small cell lung carcinomas), U-myc (unknown function), and B-myc (only identified in the mouse genome) (Cole, 1986).

#### 1.3.1.2 MYC ONCOPROTEIN FUNCTION

The c-myc oncoprotein sequence is highly conserved across chordates (van Beneden et al, 1986), with 90% amino acid identity between mice and men (Bernard et al, 1983). The protein product has a predicted molecular weight of 49kDa (Colby et al, 1982), but on SDS-PAGE a doublet of either 62/64 or 64/66kDa is observed (Alitalo et al, 1983a). The protein is post-translationally modified by phosphorylation on serine and threonine residues (Eisenman, 1985) and contains many proline residues (Abrams et al, 1982). Using cell lines, p62myc was initially immunolocalised to the cell nucleus, excluding nucleoli (Alitalo et al, 1987), and subsequently reported to be detectable in nucleus or cytoplasm depending on type and adequacy of tissue fixation (Loke et al, 1988). More recently, in the human colon and rectum, it has been immunolocalised to the cytoplasm of carcinoma cells, with only infrequent nuclear staining, but strong nuclear staining in adjacent normal epithelium, using ideally fixed specimens of colorectal carcinomas (Williams et al, 1990). In both transient and stable expression conditions, c-myc protein has been shown to bind heat shock protein 70 in the cytoplasm and induce its translocation to the nucleus (Koskinen et al, 1991).

The c-myc oncoprotein has a short half-life of 20-30 minutes, possesses DNA binding activity in vitro

(Eisenman et al, 1985; Evan and Hancock, 1985), and dissociates from DNA during metaphase in vivo (Winqvist et al, 1984). The DNA binding and protein dimerisation motifs, consisting of helix-loop-helix and leucine zipper domains, are located in the carboxy terminus of the protein, and myc forms dimers with another small nuclear protein, max, that bind to a specific hexanucleotide DNA sequence (Blackwood and Eisenman, 1991)

C-myc can prime some cells for growth competence - passage of cells from quiescence (G0) into the first phase of the cell cycle (G1) (Robertson, 1985). The levels of c-myc RNA are very low in serum-starved fibroblasts, but increase forty-fold on addition of mitogens, such as platelet derived growth factor (Kelly et al, 1983). Levels of c-myc protein do not vary in a cell-cycle dependent manner, but correlate with cell proliferation (Thompson et al, 1985; Hann et al, 1985). The precise functions of myc proteins are essentially unknown, but the available evidence points towards activity as transcription factors (Collum and Alt, 1990; Cole, 1991).

#### 1.3.1.3 MYC EXPRESSION AND ACTIVATION

Oncogenic activation of c-myc does not require mutation or other structural changes within the gene. Hence, attention has focussed on quantitative changes in myc expression in tumours. There appears to be a complex

pattern of regulation of myc mRNA at both the transcriptional and posttranscriptional levels. The c-myc promotor has two distinct transcription start sites in the first exon, containing TATAA sequences separated by 160 bp, designated P1 and P2 (Bernard et al, 1983). The second promotor is the target for stimulation by at least the adenovirus Ela protein (Lipp et al, 1989), and probably other cellular and viral transactivators. The introduction of activated c-myc and v-myc into a variety of cells results in the suppression of endogenous c-myc expression, and in rat fibroblasts this occurs at the level of transcription initiation and is proportional to the c-myc protein concentration, indicating a negative autoregulatory mechanism (Penn et al, 1990). A repressor protein (myc-PRF), found in certain cell types, interacts with a widely distributed protein, myc-CF1, and they both bind to specific short sequences just upstream of the first exon (Kakkis et al, 1989).

Serum stimulation of quiescent fibroblasts results in a transient 3 - 4 fold increase in c-myc transcription rate over 2 hours - not sufficient to account for the 20 - 40 fold increase in c-myc mRNA levels (Kelly et al, 1983). This difference is due to posttranscriptional regulation, thought to be mediated by the first exon (Cole, 1986). The very short half-life of c-myc mRNA (10-30 minutes), is due to its cytoplasmic instability. This instability is determined by sequences within the first exon. The rapid cytoplasmic turnover may be decreased by

cycloheximide treatment (Cole, 1986). Transcripts lacking the first exon are not affected by cycloheximide treatment, but c-myc RNA with only the first and second exons still have short half-lives, indicating involvement in RNA turnover of another region, in the downstream untranslated region or protein coding sequences. It is therefore of considerable interest that in the 8:14 chromosomal translocations in Burkitt's lymphomas, although the breakpoint in the c-myc containing region of chromosome 8 is variable, the second and third coding exons are separated from the first exon in a significant number of cases, and c-myc mRNA levels are higher than in equivalent quiescent cells (Cole, 1986; Lenoir and Bornkamm, 1987).

#### 1.3.1.4 CELL TRANSFORMATION BY MYC

Cell transformation by c-myc involves activation producing increased levels of functional c-myc protein. This usually results from elevated transcription (Cole, 1986). Mechanisms of activation include retroviral transduction, adjacent insertion of proviral promoters, chromosomal translocation resiting the gene to a region of influence of novel transcriptional enhancers, and gene amplification (Alitalo et al, 1987). These may result in either constitutive expression or elevation of transcription of c-myc.

Land et al (1983) used primary rodent fibroblasts to

demonstrate that c-myc had neither immortalising nor transforming activity unless linked to a transcriptional enhancer to drive its expression. Immortalisation of primary cells was induced by c-myc activated in this way, mimicking in vitro establishment of cell lines. Transfection of activated c-myc into established cell lines induced the tumorigenic phenotype, TGF production and anchorage-independent growth, with little apparent alteration in morphological appearance (Keath et al, 1984; Pragnell et al, 1985; Spandidos, 1985). Malignant transformation of rat fibroblasts, by chimaeras of myc oncoprotein and steroid receptors, was shown to be tightly oestrogen dependent and reversible on hormone withdrawal, clearly demonstrating the direct oncogenic effect of high levels of functional myc and the requirement for continued expression of myc activity for maintenance of the transformed phenotype (Eilers et al, 1989). Cells transformed by activated myc oncogenes display loss of normal growth regulation by factors such as PDGF in serum (Campisi et al, 1984). Some of the phenotypic changes induced by c-myc activation appear to be cell type specific.

#### 1.3.1.5 MYC ACTIVATION IN TUMOURS

Activation of members of the myc oncogene family in authentic human cancers occurs in a wide range of cell lineages, including haematological, neuroendocrine, mesenchymal and epithelial. These include the most common



cancers worldwide (ie cancers of the stomach, lung, liver, breast, colon, cervix, and oropharynx) (Cole, 1986; Kato et al, 1990; Field and Spandidos, 1990). Three of the best studied examples are (1) Burkitt's lymphoma (c-myc translocation to Ig loci), which illustrates myc activation as part of multifactorial carcinogenesis with Epstein-Barr virus infection of patients in the African malaria zone (Taub et al, 1982; Cole, 1986; Lenoir and Bornkamm, 1987); (2) neuroblastoma (N-myc); and (3) small cell lung cancer (L-myc and N-myc), in both of which there is myc amplification and/or elevated expression, which correlates with poor prognosis (Brodeur et al, 1984; Field and Spandidos, 1990). However, there are few tumours of any type in which myc expression is not increased to some degree.



### 1.3.2 RAS ONCOGENES

#### 1.3.2.1 RAS GENE STRUCTURE AND FAMILY

The first human cellular oncogene to be cloned (c-Ha-ras1 from the T24 human bladder carcinoma cell line) was found to be homologous to the transforming sequence of the Harvey rat sarcoma retrovirus. Similarly c-Ki-ras2 is the cellular proto-oncogene homologous to the Kirsten rat sarcoma retrovirus transforming gene. The ras oncogene family includes a third functional proto-oncogene member, N-ras, without a viral homologue. There are also two pseudogenes (Ha-ras2, Ki-ras1) (Barbacid, 1987; Spandidos, 1988).

The ras genes span 4.5 kb (Ha-ras1) and 40 kb (Ki-ras2) as a result of large differences in the sizes of their introns, which are widely divergent in their sequence composition. The Ha-ras1 gene contains one 5' non-coding exon (Ia) and four coding exons (I - IV) which synthesise a 1.2kb mRNA. Ki-ras2 has a similar arrangement of one non-coding and four coding exons, except that there are two alternative fourth coding exons (IVa and IVb) and two mRNAs are generated (3.8 and 5.5kb); although they vary in their mode of membrane attachment, specific differences in the function of the 2 alternative Ki-ras proteins have not been identified. N-ras has one non-coding, and six coding exons, with two alternative sixth exons (VIa and VIb), which result in 2.0 and 4.3kb mRNAs

(Spandidos, 1988). Although each ras gene codes for different sizes of mRNAs, they all produce the same size protein (21 kDa). The ras gene promotor regions lack TATA boxes and resemble promoters of constitutively expressed genes. They are thought to be expressed at low levels in most if not all cells, but in some differentiated cells and some tumour cells they are expressed at higher levels (Spandidos, 1988).

There are many ras-related genes in different eukaryotic organisms, which have been detected by relaxed hybridisation or specific oligonucleotide probes. These encode similarly sized proteins with varying but lower degrees of amino acid sequence homology to the ras family, indicating the existence of a wider superfamily of related genes. These include rho (present in humans and yeast), R-ras (detected in humans and rodents), and ral (in humans and primates) (Santos and Nebreda, 1989). K-rev1 (also known as rap1a, with the closely related rap1b and rap2) was discovered independently by induction of reversion of Ki-ras transformed cells (Kitayama et al, 1989), and by low stringency hybridisation with the DRAS3 gene of *Drosophila* (Pizon et al, 1988). There is a group of at least four rab genes, with homologues in yeast (YPT1), rat (BRL-ras), and humans (rab2), and several other ras-related genes (eg mel), which have not been fully characterised in terms of function.

The ras family gene sequences are highly conserved across

many species, human ras being able to support growth in mutant yeast (DeFeo-Jones et al, 1985). Ras and ras-related members of the superfamily, show a high degree of conservation of four amino acid motifs involved in guanine nucleotide binding. All ras genes also show significant conservation in another region (AA 32-40), called the "effector" region (see below), but this is shared by only some of the ras-related genes, indicating the relative functional importance of these for ras activity (Santos and Nebreda, 1989).

#### 1.3.2.2 RAS ONCOPROTEIN FUNCTION AND ACTIVATION

The ras genes encode 21 kDa proteins (p21) consisting of 188-189 amino acids, which are attached to the internal surface of the cell membrane via covalently linked fatty acids (Sefton et al, 1982). Ras proteins have several similarities to G proteins, in that they bind GTP/GDP, and show GTPase activity. There is striking conservation of structure and mechanism among different GTPases, which include (a) p21ras oncoproteins, (b) GTPases used in ribosomal protein synthesis (eg bacterial elongation factor EF-Tu), and (c) alpha subunits of the heterotrimeric signal-transducing G-proteins (Bourne et al, 1990 and 1991). The 3 classes mediate a common GTPase cycle, that acts as a molecular switch for diverse cell functions. This cycle consists of 3 conformational states. (1) Release of bound GDP converts the "inactive" state into a transient "empty" state (2), during which

GTP is more likely to bind than GDP due to differences in cytoplasmic concentration. (3) GTP binding converts it to an "active" conformation, which reverts to the GDP-bound inactive form following hydrolysis of GTP. Many GTPases have low intrinsic rates of GDP release and GTP hydrolysis, which are increased by guanine nucleotide release proteins (GNRPs) and GTPase activating proteins (GAPs) respectively. Crystalline structures of p21ras proteins and the guanine nucleotide binding domain of EF-Tu are remarkably similar, and demonstrate the conformational changes (Jurnak et al, 1990).

Five regions of the ras p21 proteins (G1-G5) delineate the guanine nucleotide binding site and mediate specific functions in the GTPase cycle. These were identified by homology with G proteins and ras-related proteins, mutagenesis of specific sites, and crystallographic data (Santos and Nebreda, 1989; Bourne et al, 1991). Regions G1 (amino acid positions 10 - 17), G2 (32 - 40), and G3 (53 - 62) interact with the phosphate groups. The G4 region (112 - 119) and G5 region (143 - 147), together bind the guanine ring.

Interestingly, all activating mutations detected in vivo are located around amino acids 12/13 and 59/61, and these cause a significant decrease in the intrinsic GTPase activity. Whilst ras genes with experimentally introduced mutations at codons 116/119 have been shown to possess transforming properties in vitro, such changes have not

been found in vivo (Santos and Nebreda, 1989). Activation of ras also occurs by overexpression due to increased transcription or gene amplification, but this is less common than point mutation.

There is an important quantitative discrepancy between decrease of GTPase activity and transforming potential of mutated ras proteins (Barbacid, 1987). This apparent paradox was resolved by the discovery of a GTPase activating protein (GAP), which stimulates the GTPase activity of normal ras by 100-fold with no effect on the GTPase activity of mutated ras proteins (McCormick, 1989). Mutational analysis has revealed that ras binds GAP through the "effector" region (G2 amino acids 32 - 40) of both normal and mutationally activated ras. The p120 GAP protein is phosphorylated and membrane linked when active, and when overexpressed it suppresses transformation by normal ras but not by v-ras (Zhang et al, 1990). Antigenic stimulation of T cells causes rapid activation of p21ras by a mechanism involving a reduction in GAP activity stimulated by protein kinase C (Downward et al, 1990). These data suggest that GAP acts as an upstream negative regulator of ras rather than a downstream ras effector target.

Ras p21 protein requires membrane attachment for full transforming activity. This occurs through a sequence of post-translational modifications to its C-terminal sequence Cys-A-A-X (Cys represents cysteine, A any

aliphatic amino acid, and X any amino acid). First, there is cleavage of the three C-terminal amino acids (-A-A-X), followed by carboxymethylation and polyisoprenylation (usually S-farnesylation of Ha-ras) of the cysteine, which is now at the C-terminus. Subsequently, there is palmitoylation of another cysteine, between two and five residues away (Lowy and Willumsen, 1989; Hancock et al, 1989). The major expressed form of Ki-ras (exon 4B) does not have an upstream cysteine residue, and is not palmitoylated. Instead it has an adjacent string of six amino acids with anionic side chains that form ionic bonds with membrane head groups. Two related inhibitors of mevalonate and polyisoprenoid synthesis, compactin and mevinolin, have been shown to inhibit polyisoprenylation of Ha-ras, thus preventing both membrane attachment and transformation function (Schafer et al, 1989). The hypervariable region is the only region displaying any significant sequence variation between the three ras gene family members, and across different species, and is likely to be important in the manifestation of functional differences between ras oncoproteins (Barbacid, 1987; Santos and Nebreda, 1989).

The known biochemical properties of ras proteins (GTP/GDP binding, guanosine triphosphatase activity and a degree of sequence homology to G proteins), have led to the suggestion that ras acts as a signal transducer of information from cell surface receptors (Barbacid, 1987;



Spandidos, 1988; Santos and Nebreda, 1989; Bourne et al, 1990 and 1991). One model of ras function begins with interaction of ligand and the extracellular component of a transmembrane receptor causing a signal to be transmitted by an unknown mechanism, perhaps conformational change, to the intracellular component. This in turn sets off a cascade of reactions. Initially, the receptor (acting as a GNP) mediates release of bound GDP leading to formation of GTP-bound p21 - the "active" state. This forms a complex with GAP, through its "effector" G2 domain, and either "active" ras alone, or together with GAP, pass on the signal to other molecules. During the interaction of normal ras with GAP, there is hydrolysis of bound GTP to GDP, returning ras to the "inactive" state. Signal-receiving molecules may include phosphoinositidases that hydrolyze phosphatidyl inositol biphosphate to form diacylglycerol and inositol triphosphate (Pichon et al, 1988; Alonso et al, 1988; Spandidos, 1988). Both of these act as second messengers, stimulating activation of protein kinase C and calcium release from intracellular stores respectively. Activated PKC increases intracellular pH through the action of Na<sup>+</sup>/K<sup>+</sup>-activated adenosine triphosphatase, and increased calcium and pH have been associated with stimulation of DNA synthesis.

The function of ras varies with species and cell type. In yeast, ras mediates survival (Katoaka et al, 1984) and mating (Fukui et al, 1986). The precise molecular



functions of yeast ras proteins are unknown, but they have been shown to influence growth by acting as positive regulators of adenylate cyclase (Barbacid, 1987; Tamanoi, 1988). In mammals the cellular effects of ras have been related to differentiation in some cell lineages, eg the rat phaeochromocytoma cell line PC12 which develops neurite outgrowth and neuron-like morphology (Noda et al, 1985; Bar-Sagi and Feramisco, 1985), and cell proliferation in others (Mulcahy et al, 1985), for example ras transcription increases in regenerating liver cells (Goyette, et al, 1983). Stimulation of the antigen receptor of T lymphocytes, produces rapid activation of p21ras via downregulation of GAP (Downward et al, 1990).

#### 1.3.2.3 CELL TRANSFORMATION BY RAS

Transformation of cells in vitro by activated ras oncogenes has been extensively studied (Barbacid, 1987). Transfection of mutated ras into immortalised rodent cells induces transformation in a dominant fashion, with higher levels of expression increasing the transforming potency of the gene (Huang et al, 1981). Non-mutated ras proto-oncogenes, however, can transform primary rodent cells only in co-operation with an oncogene of the nuclear class (eg c-myc), or if all the surrounding normal cells have been eliminated (Spandidos and Wilkie, 1984; Dotto et al, 1985; Pozzatti et al, 1986) such as by cytotoxic selection of transfectants. Activated ras

oncogenes have immortalizing activity as well as activity at later stages in transformation (Spandidos and Wilkie, 1984). Full tumorigenic conversion of primary cells generally requires additional changes (Barbacid, 1987), but can be achieved by high expression, via linkage to a transcriptional enhancer, of a mutationally activated ras (Spandidos and Wilkie, 1984). The ras oncogenes have been strongly implicated as promoting tumour progression (Vousden and Marshall, 1984) and their activation has been related to increased aggressiveness of the transformation phenotype (Marshall et al, 1985).

The role of ras oncogenes in metastasis has been correlated with the ability of ras-expressing cells to escape from a primary tumour site rather than ability to colonise a secondary site (Vousden et al, 1986). The metastatic phenotype is expressed within a few cell divisions of ras transfection (Muschel et al, 1985; Thorgeirson et al, 1985). One feature which might reduce the effects of this altered phenotype is the increased susceptibility of ras-transformants to NK cells (Johnson et al, 1985). Ras-transformed rodent cells demonstrate the features of morphological transformation such as growth of cells in foci, with overlapping of adjacent cells, and a more spindle and refractile appearance (Pragnell et al, 1985; Marshall et al, 1985). Overexpression of ras oncogenes also promotes anchorage-independent growth and stimulates TGF production (Ozanne et al, 1982; Anzano et al, 1985; Pragnell et al, 1985;

Spandidos, 1985). Ras oncogenes induce release of cells from the requirement for trophic stimuli (Kasid et al, 1985; Zahn and Goldfarb, 1986), and ras-transformed rodent cells have increased levels of expression of endogenous c-fos and c-abl oncogenes (Wyllie et al, 1987); these are features which may plausibly contribute to autonomous growth of tumour cells.

#### 1.3.2.5 RAS ACTIVATION IN TUMOURS

Mutational activation of ras has been described in 10-20% of human cancers studied (Williams et al, 1985; Barbacid, 1987), and in some there is over-expression of ras as well (Slamon et al, 1984; Barbacid, 1987), including some of the most common cancers, with correlation of ras overexpression and poor prognosis in four (stomach, breast, colorectum, and cervix, but not lung and oropharynx) (Field and Spandidos, 1990). Interestingly, overexpression of cellular ras proto-oncogenes induces only a weakly transformed phenotype in vitro (Pragnell et al, 1985).

In some human cancers (eg carcinoma of the cervix) mutation of one ras allele is sometimes found with loss of the other allele (Riou et al, 1988). In a mouse model of skin cancer, in which carcinogen-induced initiation occurs by Ha-ras mutation, followed by phorbol ester stimulated promotion, the progression of invasive squamous cell carcinoma to the more aggressive spindle

cell cancer was associated with loss of normal ras allele and imbalance of Ha-ras alleles (Quintanilla et al, 1986; Buchmann et al, 1991). This suggests a possible function for normal ras product as a competitor or partial suppressor of the mutant protein, perhaps via interactions with GAP or the downstream ras target. Transforming ras oncogenes have been described in 70% of chemically-induced tumours in various animal models (Spandidos, 1985), and direct application of Harvey rat sarcoma and BALB murine sarcoma virus v-ras genes replaces the need for chemical carcinogens to initiate two-stage mouse skin carcinogenesis (Brown et al, 1986; Quintanilla et al, 1986).

Activated myc and ras oncogenes have synergistic effects on the malignant transformation of primary cells (Land et al, 1983). This is consistent with the requirement for multiple viral oncogenes in DNA tumour virus mediated transformation of primary cells, and is the basis of the concept of separate classes of oncogenes - nuclear and cytoplasmic - and of separate oncogenes for the different stages of cell transformation (Weinberg, 1985). Hypothetical schemes of shared transformation pathways involving many oncogenes have been derived from blocking experiments using antibodies or RNA antisense methods to inhibit oncogene activities or using oncogene-revertants (eg Krev1) (Herrlich and Ponta, 1989). Although the situation is undoubtedly more complex than originally envisaged, multiple genetic events are consistent with

the multistep nature of carcinogenesis, and these may include activation of cellular oncogenes, inactivation of cellular tumour suppressor genes, and introduction of viral transforming genes.

Ras genes are known to cooperate with human papillomaviruses in transforming primary cells (Crook et al, 1988; Storey et al, 1988), and have been found to be activated in human cervical carcinomas containing these viruses (Riou et al, 1984 and 1988). The effects of ras on the growth kinetics of transformed cells is to drive rapid growth, both in vitro (Spandidos and Wilkie, 1984; Spandidos, 1985) and in vivo (Wyllie et al, 1987). For these reasons, the ras oncogene represents an interesting candidate that may have effects on tumour cell apoptosis, either alone, in combination with another oncogene of a different class such as the myc oncogene, or with human papillomavirus transforming genes.

#### 1.4 HUMAN PAPILLOMAVIRUSES AND CERVICAL NEOPLASIA

Epidemiological studies of cervical neoplasia have suggested a direct causal relationship with sexual activity (Munoz et al, 1987 and 1991). Early onset of sexual activity and multiple sexual partners - apparently acting as independent variables - are the principle risk factors (Koss and Phillips, 1974; Sebastian et al, 1978). A further risk factor is exposure to the "high risk male", characterised by a history of promiscuous sexual activity and/or exposure to contraction of genital neoplasia (Kessler, 1977; Graham et al, 1979; Cartwright and Sinson, 1981). Over the past twenty years the search for venereally transmitted carcinogens has included components of semen and various viruses (Vonka et al, 1984 and 1987). HPVs have emerged as the most promising candidates.

Human papillomaviruses have been implicated in the genesis of several benign and malignant tumours of the cervix, vulva, and anogenital region (zur Hausen, 1977 and 1980; Pfister, 1984 and 1987; Arends et al, 1990b). The inability to propagate papillomaviruses in cell culture hampered research into their oncogenic potential until the advent of molecular cloning of viral DNA in the early 1980's. This allowed labelled viral DNA probes to be used to investigate the prevalence of viral DNA in various tumours and led to the cloning and characterisation of new papillomavirus types. DNA

sequencing of different viral types and manipulation of whole viral genomes and subgenomic fragments in experimental model systems has shed light on the molecular biology of many viral functions including malignant transformation.

Definitive investigations to prove a causal role for this group of viruses in genital neoplasms are currently lacking (anonymous Lancet editorial, 1985; zur Hausen, 1989). There are, for example, insufficient long term follow-up studies on matched case and control cohorts using sensitive methods of detection of HPV. In this section the association of HPVs with cervical neoplasia, the biology of HPV infection, the genomic organization and gene functions of papillomaviruses are reviewed. One possible model of carcinogenesis is presented, which accounts for clinical and experimental data. The morphological basis of cervical neoplasia and the involvement of HPVs are discussed in chapter 2, and cell transformation by HPVs in chapter 3.

#### 1.4.1 PAPILLOMAVIRUS TYPES

Papillomaviruses are members of the Papovaviridae family and are composed of capsids containing closed circular double-stranded DNA of approximately 8 kb in length (Pfister, 1984). They are classified by the host species infected and by the degree of DNA cross hybridization in the liquid phase: designation of

different types requires less than 50% cross hybridization (Coggin and zur Hausen, 1979). A change in classification criteria was proposed at the 1991 International Papillomavirus Workshop to a system based on sequence homology within specific viral genes. Over 60 types of HPV have been identified using existing criteria, some being further divided into subtypes (greater than 50% cross hybridization but with differing restriction enzyme cleavage patterns, eg HPV 6a, 6b, 6c). Various types of HPV grouped according to the sites of associated lesions and overall sequence homology determined by hybridization are shown in table 1.2 (Pfister et al, 1986; Pfister, 1984, 1987a and 1987b; deVilliers, 1989). A phylogenetic tree, constructed from computer alignment of DNA sequences of the highly conserved E1 C-terminal domain (Giri and Danos, 1986), suggested three papillomavirus families: genital, skin and fibropapillomaviruses. Fibropapillomaviruses seemed to have evolved separately from the other two families.



**TABLE 1.2****HUMAN PAPILLOMAVIRUS TYPES INFECT DISTINCT SITES**

SITE	GROUP	HPV TYPE
Skin	A I	1
	A II	2, 3, 10, 27-29
	A III	4
	A IV	7
	A V	26
EV	B I	5*, 8*, 9, 12, 14, 15, 17, 19, 20-25, 36-38, 46, 47, 49
	B II	50
Mucosa	C I	6, 11, 13, 44
	C II	16*, 31(*)
	C III	18*, 32, 42, 45(*)
	C IV	30
	C V	33(*)
	C VI	34
	C VII	35(*)
	C VIII	39(*)
	C IX	41
	C X	43

Classification of the first 50 HPV types (except HPV 40 and 48) by site of infection and overall DNA sequence homology, determined by cross hybridisation (data modified from previous classifications by Pfister, 1987; deVilliers, 1989). The sites of viral infection are: (A) skin, (B) skin of epidermodysplasia verruciformis patients (EV), and (C) various mucosae and specialised skin (cervix, vulva, anogenital region, and restricted regions of the upper respiratory tract). HPV types strongly associated with malignant tumours are indicated by \* and those found in premalignant and occasional malignant lesions by (\*). Such a classification based on overall sequence homology may be misleading, as different HPV types may show similarity in some parts of the viral genome, such as those encoding capsid proteins, but disparity in other parts controlling important biological functions including transformation.

#### 1.4.2 BIOLOGY OF PAPILLOMAVIRUS INFECTION

The striking feature of different human papillomavirus types is their predilection for infection of squamous epithelia at specific sites (table 1.2). Although such site specificity is not fully understood, it may be explained by attachment of viral capsid proteins to specific surface receptors on epithelial cells. Alternatively, there may be close linkage of the site-specific epithelial differentiation programme with permissiveness for viral replication, perhaps through cellular regulatory proteins binding to viral DNA at transcription control sequences.

Papillomavirus infection of squamous epithelia produces epithelial proliferation with different patterns of growth depending on the site and HPV type. In benign infections the incubation period may vary from a few weeks to several months (Rowson and Mahy, 1967). Some lesions may regress spontaneously, whilst others persist or progress.

It is thought that the virus primarily infects epithelial basal cells at the site of a microabrasion or by direct contact with exposed basal cells, such as occurs at the transformation zone of the cervical squamo-columnar junction (90% of HPV infections of the cervix occur at this site). The viral replication cycle appears to be linked to the

differentiation process of the epithelium (Bedell et al, 1991), and there is evidence that HPV can modify keratinocyte maturation (Brescia et al, 1986). Early viral genes are expressed in the lower epithelial layers, and regulate viral DNA synthesis, whereas late viral genes which encode capsid proteins are expressed in the upper epithelial layers (Pfister, 1987; Galloway and McDougall, 1989). Following replication, viral DNA is sufficiently abundant within nuclei in intermediate and superficial layers that it can be demonstrated by in situ hybridization. Similarly synthesis of structural viral proteins in superficial epithelial cells can be demonstrated by immunocytochemistry. HPV infection of cervical, anogenital and other epithelia often produces koilocytotic change in the intermediate and superficial layers (Koss and Durfee, 1956). Koilocytosis is characterized by hyperchromatism and crenation of nuclei with perinuclear clearing of the cytoplasm, is often associated with multinucleation of infected cells, and is considered to be an HPV-induced cytopathogenic effect. Some but not all koilocytes contain capsid antigens or virion particles (Morin et al, 1981; Casas-Cordero et al, 1981), thus the koilocyte represents a marker of HPV infection, but not necessarily of complete viral maturation. Other histological features sometimes associated with HPV infection include individual cell keratinisation, multinucleation, parakeratosis, acanthosis, and

papillomatosis (Meisels et al, 1982).

#### 1.4.3 PAPILLOMAVIRUSES AND THE CERVIX

Detail of the relationship between HPVs and cervical neoplasia is given in the introduction to chapter 2, but certain principles require mention here. Most information relating to the oncogenic potential of HPVs has been gained by investigating viral prevalence in neoplastic lesions of the cervix. Low risk HPV types 6b and 11 are associated with cervical condylomas and CIN 1, whereas high risk HPV types 16 and 18 are found in up to 80% of CIN 2 and CIN 3 lesions, and up to 100% of invasive cervical cancers (Gissmann, 1984; de Villiers et al, 1987; Stanley, 1990; reviewed by Arends et al, 1990). Small proportions of cervical neoplasms contain other HPV types such as 30, 40, 42, 43, 44 and 58 in benign lesions, and types 31, 33, 35, 39, 45, 51 and 52 in both CIN and cancers (Lorincz et al, 1986, 1987a and 1987b; Beaudenon et al, 1986 and 1987; deVilliers, 1989). However, HPVs are also detectable in control populations (Toon et al, 1986; de Villiers et al, 1987; Melchers et al, 1989), indicating that other events are required for development of malignancy.

#### 1.4.4 SYNERGISM OF PAPILLOMAVIRUSES WITH OTHER RISK FACTORS

Zur Hausen (1982) suggested that in the development of cervical neoplasia, papillomaviruses may act as

promotor-like agents in synergism with carcinogenic initiators such as cigarette smoke, or herpes simplex virus (HSV). Several epidemiological studies have revealed a low but consistent increase in relative risk due to heavy or prolonged smoking, and a probable increase in risk from prolonged use of combined oral contraceptives (Vessey, 1986). Access to cervical epithelium by cigarette smoke products is indicated by the presence in cervical mucus of tobacco metabolites, which may be metabolised to carcinogens such as nitrosamines (Sasson et al, 1985).

HSV has been shown to possess mutagenic and carcinogenic properties in infected cells (zur Hausen, 1982; Vonka et al, 1987). Although HSV may have the potential to act as a hit-and-run initiator, the lack of persistent viral DNA, RNA or protein in most neoplastic lesions and the equivocal findings in a prospective study of HSV in cervical cancers (Vonka et al, 1984) do not support a key pathogenic role for HSV in genital neoplasia.

Studies on renal allograft recipients, treated with immunosuppressants to inhibit graft rejection, have shown a much higher incidence of CIN compared to controls, and HPV 16 or 18 DNA has been demonstrated in most lesions (Alloub et al, 1989). This supports the hypothesis that immunosuppression may play a part in CIN development.

#### 1.4.5 PAPILLOMAVIRUS GENOME ORGANIZATION AND FUNCTION

The pathology associated with HPV infection requires some description of genome organization, gene functions, and regulation.

##### A). Genome Organization

The nucleotide sequences of the closed circular double stranded DNA of all animal and human papillomaviruses determined to date show a remarkably similar colinear genome organization (Chen et al, 1982; Schwarz et al, 1983; Seedorf et al, 1985; Matsukura et al, 1986) (figure 1.1). All potential protein coding sequences (open reading frames) occur in similar positions on one DNA strand and are of approximately similar lengths (Baker, 1987). There is an upstream regulatory region (URR) containing the transcription control sequences and origin of replication, followed by 6 to 8 early region and 2 late region protein coding sequences (figure 1.1).

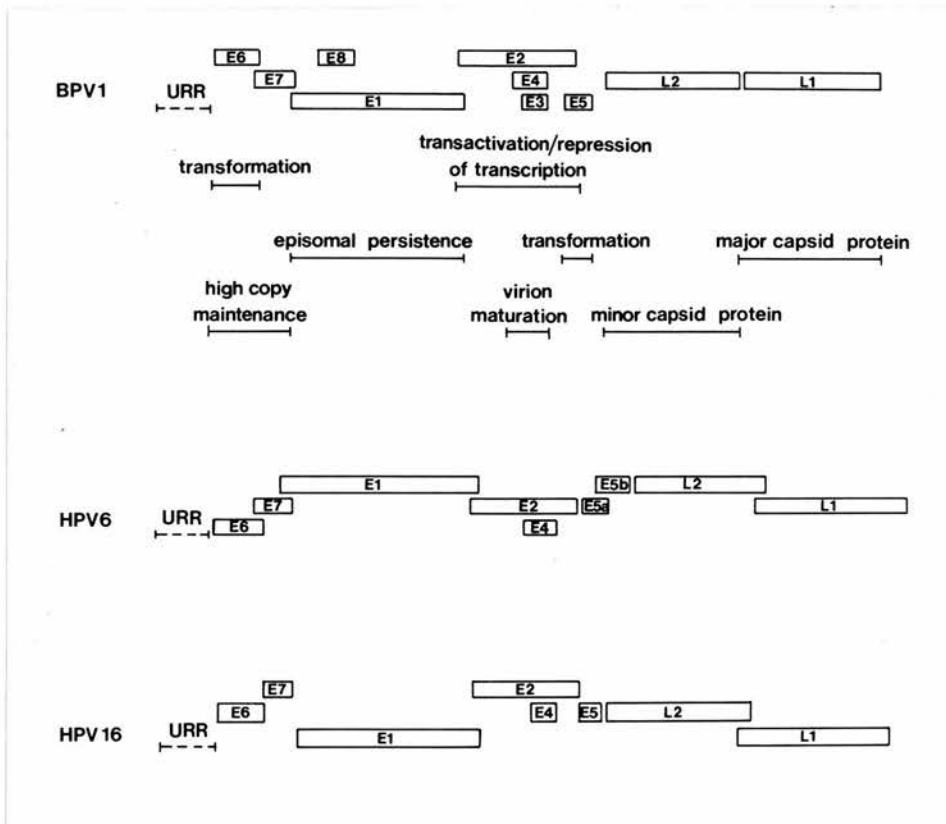


Figure 1.1

Genomic organisation of bovine papillomavirus 1 (Chen et al, 1982) with aligned sequences of HPV 6b (Schwarz et al, 1983) and HPV 16 (Seedorf et al, 1985) (incorporating the HPV 16 E1 sequence correction by Matsukura et al, 1986). The boxes represent open reading frames in the 3 possible translation frames; each open reading frame is capable of coding for a polypeptide uninterrupted by a stop codon. There is a translation start codon (ATG) at or near the beginning of almost all the coding sequences. The functions assigned to BPV 1 coding sequences by genetic analysis are shown.

## B). Gene Functions

Study of cell transformation in vitro, and of viral warts and cancers, has led to assignation of function to most of the protein coding sequences of human and animal papillomaviruses (figure 1.1). The late region genes (L1 and L2) code for structural capsid proteins (Pilacinski et al, 1984). Virion particle maturation is associated with E4 (Doorbar et al, 1986). Cell transformation functions map to the early region sequences E6 and E7 for HPV, and E6 and E5 for BPV (Schiller et al, 1984; Yang et al, 1985; Munger et al, 1989a and 1989b; reviewed by DiMaio, 1991): E6 binds to the p53 tumour suppressor gene product, E7 to the retinoblastoma protein, and E5 activates the transforming activity of both epidermal and platelet-derived growth factor receptors (Phelps et al, 1988; Martin et al, 1989; Munger et al, 1989b; Werness et al, 1990; DiMaio, 1991). A detailed discussion of HPV genes involved in cell transformation is provided in chapter 3.

Episomal persistence maps to E1 (Lusky and Botchan, 1985). E2 encodes at least two regulatory proteins which bind to specific sequence motifs in the URR (Spalholz et al, 1985; Lambert et al, 1987). Full length E2 protein upregulates transcription of other viral genes by binding and activating a conditional enhancer in the viral URR. This consists of at least two tandem repeats of the ACCG-N4-CGGT palindrome, or sometimes the more degenerate ACC-N6-GGT sequence (Phelps and Howley, 1987).



The other E2 product is a transcriptional repressor encoded by the carboxy half of the E2 gene (E2Tr), which binds to the same sequence and competitively inhibits the larger transactivator protein (Chin et al, 1988). The exact mechanism of control of early gene transcription by E2 is not fully understood, but the levels of the two competing proteins may determine the overall effect (Ward et al, 1989). The usual balance of E2 products causes repression in warts, whereas disruption of the E2 gene by integration or deletion at this site as occurs in cancers, would be expected to abrogate repression and produce uncontrolled expression of E6 and E7 (Ward et al, 1989). Fusion products containing E2 sequences and regions of other open reading frames may also contribute to regulation of gene expression. It is theoretically possible that E2 products may also regulate expression of some host cell genes with similar E2-responsive enhancer elements. The URR also contains E2-independent enhancer elements that are cell type-specific and glucocorticoid and progesterone responsive (Cripe et al, 1987; Gloss et al, 1987 and 1989; Pater et al, 1988a; Gius et al, 1988; Chan et al, 1989), implying complex control of expression of viral early and late genes by cell type-specific, hormonal and viral factors.

#### C). Transcription of papillomaviruses

Different patterns of HPV gene expression are found in

replicative infections compared with cancers. In condylomas a complex pattern of RNA splicing occurs (Pettersson et al, 1987). Two or three main promoters are used for initiation of transcription, one just before the start of the E6 gene, and the others at or just after the start of the E1 gene. There is a poly A addition site at the end of the early region genes, and another at the end of the late region genes. In replicative infections, early region genes are expressed abundantly in parabasal cells and in the middle of the epithelial layer, whereas late gene expression is seen in the superficial keratinocytes. Control of this change is not due to a promoter switch mechanism, as seen with some viruses, but rather to read-through of the early poly A signal (Galloway and McDougall, 1989). In cancers, the E6 and E7 sequences are the most abundantly expressed, and are often the only viral genes to be expressed (Schwarz et al, 1985; Shirasawa et al, 1987; Smotkin and Wettstein, 1987). This reflects structural alterations to the viral genome due to integration of HPV DNA in many cancers, as described below.

#### 1.4.6 PHYSICAL STATUS OF VIRAL DNA IN TUMOURS

Important differences in the physical status of HPV DNA have been demonstrated in benign and malignant tumours. HPV 6b and 11 are maintained as extrachromosomal circular DNA episomes in benign cervical lesions, whereas HPV 16 and 18 have been found to be

integrated in most human cervical carcinomas and carcinoma-derived cell lines (Lehn et al, 1985; Pfister, 1987b; Choo et al, 1987). Integration occurred in 72% of 40 carcinomas containing HPV 16, and 100% of 23 cancers containing HPV 18 (Cullen et al, 1991). In a series of 27 premalignant cervical lesions (CIN 1 to CIN 3), 66% contained HPV 16 DNA; in mild dysplasias HPV 16 DNA was found only as extrachromosomal episomes, but in all higher grades of dysplasia integration of the viral DNA was detected (Lehn et al, 1988). However, in another study only 3 (all CIN 3) of 100 CIN biopsies showed integration (Cullen et al, 1991).

Carcinomas may contain one copy of integrated viral DNA or multiple head-to-tail tandemly repeated copies, sometimes at many host cell genome integration sites with possible disruption of cellular genes at those sites (Popescu et al, 1987; Shirasawa et al, 1987; Popescu and DiPaolo, 1989 and 1990). Some carcinomas have both integrated and episomal viral DNA, some episomal only, and some integrated only (Matsukura et al, 1989). As proof of integration Durst et al (1985) have cloned DNA fragments of virus-host genome junctions. Viral sequences are often amplified together with cellular flanking sequences (Wagatsuma et al, 1990).

The cause of viral integration is not known, but it may be due to a carcinogen-induced recombination event or perhaps an intrinsic property of certain virus

types. Some analyses of integration sites in the host genome have indicated that integration sometimes occurs near cellular oncogenes or at fragile sites (Durst et al, 1987a; Popescu and DiPaolo, 1989 and 1990). However, many studies show integration occurring randomly in the genome (Smith et al, 1989). The pattern of viral DNA integration into the host genome in carcinomas is monoclonal suggesting that this occurs prior to expansion of the malignant clone of cells.

Essentially similar findings derive from experimental reconstructions. Human genital keratinocytes immortalized in vitro by HPV types 16, 18, 31 or 33 contain integrated and transcriptionally active viral genomes, whereas cells transfected with HPV types 6b or 11, contain only episomal viral DNA and have the same lifespan as nontransfected cells (Woodworth et al, 1989). HPV 16 DNA integration frequently occurred at fragile sites, at the junction of chromosome translocations, at achromatic lesions, and within homogenously staining regions and duplicated segments (Popescu and DiPaolo, 1990). This was sometimes associated with the induction of chromosome alterations, such as marker chromosomes.

In both cervical carcinomas (Lehn et al, 1985; Choo et al, 1987) and cell lines derived from them (Schwarz et al, 1985; Pater and Pater, 1985; Shirasawa et al, 1987) there is a consistent pattern of disruption of the

circular viral DNA upon integration. The recombination event frequently occurs within E1 or E2, and less often L2, sometimes causing focal deletions (figure 1.2) (Schwarz et al, 1985; Pater and Pater, 1985; Choo et al, 1987). This pattern of DNA interruption provides clues to the means whereby viral genes disturb growth properties of host cells.

Viral integration by recombination within the E1 or E2 genes leaves E6 and E7 directly coupled to the viral enhancer and promotor sequences in the URR, thus allowing their continued expression after integration. Disruption of E1 or E2 during integration inactivates or decouples the other early and late viral genes from the viral promotor and enhancer sequences in the URR. Viral genes and functions lost include E1 (responsible for episomal persistence), E2 (producing loss of both positive and negative control of viral transcription), E5 (with possible changes in growth modulation) and the late genes L1 and L2 (stopping synthesis of capsid proteins which might otherwise present possible targets for immune recognition in affected cells). A cervical carcinoma containing episomal HPV 16 with a deletion of a large portion of the URR has been described (Tidy et al, 1989c), which also implicates alteration of viral gene expression as a possible contributor to the neoplastic process.

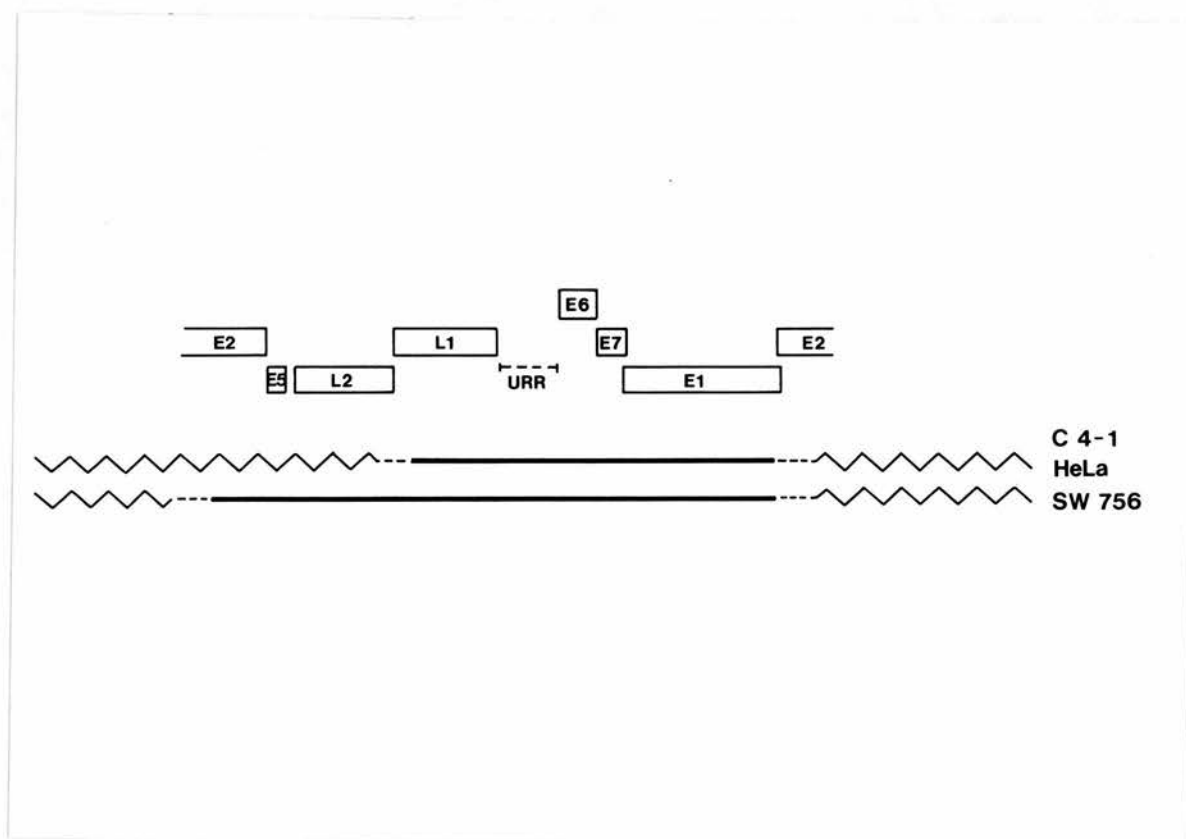


Figure 1.2

Pattern of integration of HPV 18 DNA in three cervical carcinoma cell lines by disruption of the circular viral genome at the E1-E2 region. The putative transforming sequences E6 and E7 adjacent to the viral promoters and enhancers in the upstream regulatory region (URR) remain intact, whilst the other viral coding sequences are either destroyed or decoupled from the URR. Wavy lines represent host cell DNA. Data redrawn from Schwarz et al (1985).

Another consequence of viral integration at the E1 - E2 region is the juxtaposition of HPV E6 - E7 DNA and host cell DNA with uncoupling of the viral signals that usually terminate transcription. Termination of the integrated E6-E7 genes would then be achieved through use of cryptic signals in the adjacent host DNA (Durst et al, 1985) (figure 1.2). This leads to the formation of RNA fusion transcripts (HPV E6-E7 encoded RNA linked to RNA transcribed from adjacent host cell DNA). Theoretically such fusion transcripts with changes in the RNA secondary structure could have altered stability producing increased half life or translatability of the E6 - E7 coding mRNA, with increased or sustained E6 and E7 protein activity. Schneider-Manoury et al (1987) studied a keratinocyte cell line derived from a vulval Bowenoid papule, in which HPV 16 DNA had integrated with disruption and deletion within the E2-L2 sequences. Further, expression of the E6 and E7 genes was demonstrated. The integration event was shown to have occurred in the original (pre-malignant) lesion. Thus, HPV DNA integration may represent a critical event in the progression of genital neoplasia, by inducing uncontrolled expression of the viral genes E6 and E7, possibly in a continuous way.

#### 1.4.7 PAPILLOMAVIRUSES AND HOST CELL GENETIC CHANGES

The hypothesis that high risk HPVs play a contributory role early on in cervical neoplasia, is supported by much of the data presented in previous sections. However, other genetic changes within the host cell appear to be required for the full development of a malignant neoplasm capable of invasion and metastasis. Several candidates for such changes have been studied, and these will be reviewed in the ensuing paragraphs. HPV E6 and E7 interact directly with the p53 and Rb tumour suppressor proteins (Phelps et al, 1988; Werneess et al, 1990), and perhaps other tumour suppressor genes may be involved. Of the possible oncogenes which may become activated in cervical cancer, myc and ras have been most extensively studied. A different class of host genes appears to regulate HPV gene expression. Such host cell genetic changes may occur as mutations, alterations in gene dosage, gene inactivation, or gene loss, and some of these changes may be brought about by the development of tumour cell aneuploidy.

Allele loss in cervical cancer has not been extensively investigated. One preliminary study suggested that only a small proportion of tumours lost alleles at those loci that are most frequently lost or altered in other common solid malignancies such as breast or colon (Busby-Earle, personal communication). Neither loss nor rearrangement of the p53 or Rb1 loci were observed in a series of 50



squamous cell carcinomas of the anus, of which 76% were positive for HPV 16 DNA and 8% for HPV 18 DNA (Crook et al, 1991). However, analysis of the p53 DNA sequence in 9 cases showed p53 mutations in 3 tumours negative for HPV, and only wild type sequence in 6 HPV positive cancers. This supports the hypothesis that loss of wild type p53 function is important in the development of anogenital cancers, and that this may be brought about either by E6 complex formation with p53 protein in HPV containing tumours, or by mutation in HPV negative cancers.

Most studies on oncogene activation in cervical cancers, have focussed on myc and ras in established invasive genital carcinomas and some carcinoma derived cell lines. Resected specimens of human cervical carcinomas containing HPV 16 or 18 DNA, show a correlation between amplification of both c-myc and c-Ha-ras1 and advanced tumour stage (stages III and IV) (Riou et al, 1984). In early (stage I) invasive cervical carcinomas c-myc expression is closely correlated with prognosis, with the 18 month relapse-free survival rates of cases with normal myc expression being 90% compared to 49% for those with high myc expression (Riou et al, 1987). In a smaller study, Ocadiz et al (1987) reported 49% cervical cancer cases had an amplified c-myc gene, and that 43% presented with both amplification and rearrangement of c-myc. In a series of 50 anal carcinomas, 15 (30%) showed amplification of c-myc, of which 13 contained HPV 16, and 1 contained HPV 18

(Crook et al, 1991).

The WC12 human keratinocyte cell line, which generates raft cultures histologically identical to CIN 1 and contains episomal HPV 16 DNA, was compared with one of its sublines with increased growth rate and resistance to differentiation stimuli. The more aggressive subline had increased c-myc expression due to amplification of the c-myc locus resulting from chromosome 8q duplication (Crook et al, 1990). Studies on the sites of integration of HPV 18 DNA into the host cell genomes of both HeLa and C4-1 cervical carcinoma derived cell lines have revealed that the virus is inserted at chromosome 8q24, approximately 40 kb 5' proximal to the c-myc gene and that the myc mRNA levels are increased in both lines, suggesting cis-activation of myc by adjacent insertion of viral promotor/enhancer sequences (Durst et al, 1987). Shirasawa et al (1987) found HPV 16 and 18 DNA integrated in 5 cell lines derived from cervical carcinomas in Japanese patients; all 5 cases contained the URR and E6-E7 DNA sequences and expressed HPV specific RNA. Three of the five cases expressed c-myc and c-Ha-ras1 at about 9 times the normal levels.

Elevated expression of the ras p21 protein was demonstrated in cervical neoplasms immunocytochemically, and carcinomas had a higher staining intensity than premalignant lesions (Agnantis et al, 1988). The c-Ha-ras1 locus on chromosome 11 was analysed in a series

of cervical cancers, of which 90% contained HPV 16 or 18 DNA. One ras allele was lost in 36% of heterozygous tumours and mutations in ras gene codon 12 were found in 2% of early cervical carcinomas (stages 1 and 2) and 24% of advanced tumours (stages 3 and 4). 40% of tumours with a ras mutation had also lost the other allele (Riou et al, 1988). In the same series, the c-myc gene was either overexpressed or amplified in 100% of tumours containing mutations and 70% exhibiting a deletion, supporting complementation between these two oncogenes in tumour progression. Such complementation was anticipated from experiments with rodent cell fibroblasts (Land et al, 1983).

The possibility that cellular genes might control HPV expression has been studied in HeLa cells, a long established aneuploid cervical carcinoma cell line expressing HPV 18. HeLa cells lose both tumorigenicity in nude mice (Stanbridge et al, 1982) and expression of HPV 18 in vivo (zur Hausen, 1986) after fusion with normal fibroblasts. Such hybrid cells progressively lose chromosomes; in revertants which have lost chromosome 11, both tumorigenicity and HPV 18 expression in vivo return. Zur Hausen (1986) suggested that these data support the existence of a host cell gene on chromosome 11 coding for a cellular interfering factor (CIF). It is postulated that CIF is activated when the hybridoma is injected into nude mice and suppresses HPV expression

and tumorigenicity. Presumably this suppression is removed following loss of the CIF gene on chromosome 11. HPV 16 enhancer/promotor sequences have also been shown to function more strongly in del-11 cells - human fibroblasts with a deleted segment of chromosome 11 (p11 to p15) - than in normal diploid embryonic fibroblasts (Smits et al, 1990). Thus neoplastic progression could be viewed as a consequence of the breakdown in host cell control of persisting viral DNA.

Additional consequences of chromosome 11 loss may also be important in tumorigenesis. First, c-Ha-ras1 (on chromosome 11p) can be activated in a variety of tumours and cell lines by mutation of one allele and loss of the other (Spandidos, 1988; Spandidos and Field, 1990; Buchmann et al, 1991). The same pattern occurs in cervical cancers containing HPV (Riou et al, 1988). Second, there are two candidate tumour suppressor genes implicated in Wilm's tumour, on a region of chromosome 11p distinct from c-Ha-ras1 (Koufos et al, 1984). Interestingly, when HPV 16 and mutated ras were were transfected into primary human fibroblasts, the resulting fast-growing, immortalized, but non-tumorigenic cell line was found to have sustained a translocation involving chromosomes 1 and 11 (Matlashewski et al, 1988). Aberrations of chromosomes 1, 3 and 5, as well as generation of marker chromosomes have also been found in cervical carcinomas (Atkin and Baker, 1984; Atkin, 1986; Teyssier, 1989; McDougall, 1990).

Changes in gene dosage or chromosomal loss occur following the transition to aneuploidy, and this represents one possible mechanism of CIF or tumour suppressor gene inactivation, as well as contributing to oncogene activation. A role for HPV in the genesis of aneuploidy in cervical neoplasia is supported by the association of abnormal mitoses with some HPV infections (Crum et al, 1985), and the development of aneuploidy in primary human cells by transfection of HPV 16 or 18 DNA into genital keratinocytes (Durst et al, 1987b; Pirisi et al, 1987 and 1988) and fibroblasts (Matlashewski et al, 1988). The importance of aneuploidy is uncertain, but it was found in 60 - 80% of CIN 3 lesions, whereas diploid DNA content was seen in 100% of normal/CIN 1 and 90% of CIN 2 biopsies (Jakobsen et al, 1983; Hanselaar et al, 1988). The DNA ploidy pattern of concomitant invasive cancers was generally identical with adjacent CIN 3, suggesting that the cancer developed from the CIN, and that aneuploidy first occurred in the premalignant lesion (Hanselaar et al, 1988).

#### 1.4.8 PAPILLOMAVIRUSES AND CERVICAL CARCINOGENESIS

This section sets out a model of events which may occur during the transition of keratinocytes manifesting HPV replication to neoplastic cells with malignant potential (figure 1.3). This emphasises the coordinate or sequential participation of HPV and other events such as oncogene activation. Such multistage progression would explain several features of cervical cancers. The small number of invasive carcinomas in comparison with the high prevalence of HPV infection, the monoclonality of cervical carcinomas, and the long latent period required between HPV infection and development of carcinoma.

The model envisages three major phases. First, there is infection by a high risk HPV type. Various HPV types differ in their oncogenic potential in the cervix. This may be due to variations in the growth modulatory activities of their E6 and E7 proteins, perhaps combined with differences in their levels of expression. Transcription factors may also be important, both through viral proteins binding to host cell responsive DNA elements, and host factors binding to viral transcription control elements. Whatever the mechanisms, virally-mediated growth advantages may increase the probability of subsequent events occurring that bring about malignant transformation.

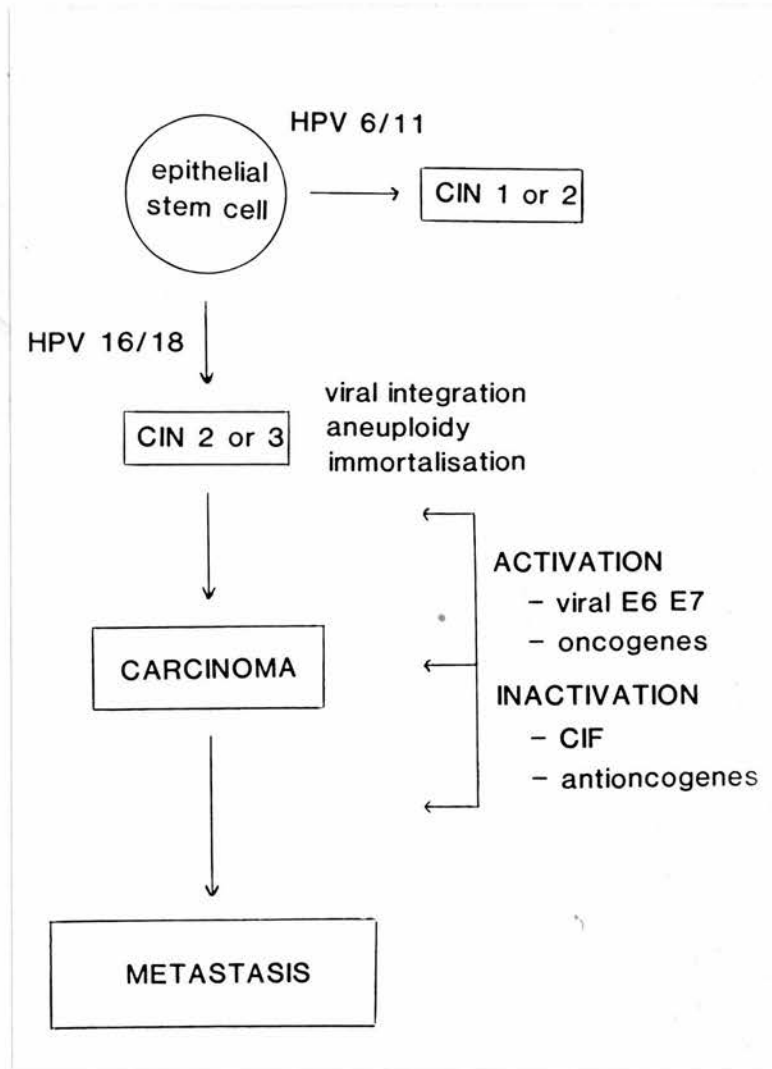


Figure 1.3

Possible events in tumour progression following HPV infection of basal stem cells in cervical epithelium, perhaps aided by immune suppression and exposure to mutagens. High risk viruses (HPV 16 and 18) produce high grade neoplastic cervical lesions in which viral integration in combination with loss of HPV-cellular inhibitory factor gene (CIF) activity cause constitutive activation of viral E7 and E6 transforming sequences. There is associated immortalisation and development of aneuploidy. Oncogene activation and antioncogene inactivation may be involved in conversion to invasive or metastatic carcinoma.

The second phase is viral integration. This has important consequences for HPV genes E6 and E7, which are conserved intact (Schwarz et al, 1985), and show evidence of persistent and possibly elevated expression in carcinomas (Shirasawa et al, 1987; Smotkin and Wettstein, 1987). E6 and E7 have proven transforming activities, and bind the p53 and Rb tumour suppressor proteins respectively (Munger et al, 1988b; Werness et al, 1990). In vitro reconstructions demonstrate that E6 and E7 cooperate with activated ras to transform primary rodent cells to malignancy (Storey et al, 1988; Crook et al, 1988).

Integration of HPV DNA also occurs following experimental introduction into human keratinocytes, and this results in the development of a cluster of growth modulation changes including reduced growth factor requirement (growth in low serum), aneuploidy and immortalization (rescue from senescence) (Woodworth et al, 1988 and 1989). These immortalized keratinocytes may be the equivalent of CIN 2 or CIN 3 in vivo as raft cultures formed by them show morphological features closely similar to high grade intraepithelial neoplasia (McDougall, 1990; Meyers and Laimins, 1992). This may lead to an expansion of a population of aneuploid, premalignant cells, promoting increased opportunity for further genetic changes.



The third phase includes progressive acquisition of host cell genetic lesions including activation of oncogenes, and inactivation of both tumour suppressor genes and cellular genes controlling viral expression (eg the C1F gene). Activation of the c-myc and c-Ha-ras1 oncogenes has been demonstrated in cervical carcinomas and cell lines derived from them; in some studies myc activation appears to correlate with early stage tumours and ras activation with late stage tumours (Riou et al, 1987 and 1988). Loss or inactivation of the putative C1F would allow further deregulation of expression of HPV E6 and E7 genes (zur Hausen, 1986).

The credibility of this model would be strengthened if two elements implied by it could be verified, and it is the aim of this thesis to do this. First, it is important to establish at what stage in the CIN-cancer sequence, and how frequently, HPVs exert their effect. Although much is already known of the prevalence of HPV in cancers, controversy surrounds the levels of prevalence in the normal population, and less is known about the association with different grades of CIN. Thus, there is a need for accurate information, which by necessity must often be derived from small tissue samples, requiring a highly sensitive and specific HPV detection assay, as described in chapter 2. Such data may also shed light on the prognostic relevance of different HPV types, in terms of progression from CIN to cancer and cancer cell type.

Second, experimental reconstructions have demonstrated oncogenic activity of HPV DNA in vitro, and this approach provides an opportunity to investigate specific phenotypic effects of interest. In chapter 3, rodent fibroblasts transfected with HPVs, ras and myc, are used to study genetic regulation of the rate of tumour cell death by apoptosis, and how important this is in influencing the overall rate of tumour growth.

## 2. PAPILLOMAVIRUSES IN CERVICAL NEOPLASIA

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## 2.1 INTRODUCTION

### 2.1.1 CERVICAL NEOPLASIA: EPIDEMIOLOGY AND PATHOLOGY

Cervical cancer is the second most common cancer worldwide, and the most common in developing countries, where screening programmes have been associated with 50% - 60% decrease in mortality (The Walton Report, 1982; WHO Meeting: control of cancer of the cervix uteri, 1986; Cotran et al, 1989). Cervical cancer is presently ranked eighth as a cause of cancer deaths in women in the United States of America, with about 7000 deaths from an estimated 13 000 new cases per year of invasive cancer, and there are also 50 000 new cases per annum of carcinoma in situ/CIN 3 (Silverberg and Lubera, 1989). Both the incidence and prevalence of CIN lesions in the USA, has increased steadily over the past three decades, particularly in teenagers and women under 30 (Berkowitz et al, 1979; Sadeghi et al, 1984). The incidence of cervical cancer has also been gradually increasing from 1978 to 1987 in Scotland (Cancer Registration Statistics Scotland, 1990).

The accessibility of the cervix has permitted extensive study of both non-invasive neoplasms - cervical intraepithelial neoplasia - and invasive cervical carcinomas. From these investigations evidence has accumulated to support the concept that cervical cancer arises from precursor neoplastic lesions (Ferenczy and Winkler, 1987; Richart, 1987). A continuum of

progressively more atypical changes of the cervical epithelium has been described. Early changes involve the appearance of atypical cells in the basal and lower layers of the squamous epithelium, with persistence of normal differentiation towards the prickle and keratinizing layers. The atypical cells are pleomorphic, show loss of polarity, changes in nucleocytoplasmic ratio and increased mitotic figures, some above the basal layer. More aggressive lesions in the continuum show progressive involvement of more layers of the epithelium with less surface differentiation. In the most advanced lesions, the full thickness of the epithelium is replaced by atypical cells. Lesions within this spectrum of morphological change have been classified by three systems.

(1) The classic terminology separates non-invasive cervical epithelial lesions into two histologically distinguishable groups: carcinoma in situ (CIS) and dysplasia. Dysplasia is subdivided into mild, moderate or severe according to the proportion of the epithelium involved by atypical cells; full thickness involvement is known as carcinoma in situ (Weid, 1961).

(2) Cervical intraepithelial neoplasia (CIN) is a term originally introduced to emphasize the continuous nature of the change, and is subdivided into three grades depending on the number of thirds of the epithelium involved by atypical cells (Barron and Richart, 1968;

Richart, 1973).

(3) The most recently introduced descriptive term is squamous intraepithelial lesion (SIL), which may be subdivided into high and low grades (National Cancer Institute Workshop: The 1988 Bethesda System, 1990). This system was designed primarily for use in reporting cervical smears, and was intended to describe the squamous cell precursors to invasive squamous carcinoma, whilst incorporating and superceding CIN, dysplasia, and CIS. The phrase "cellular changes associated with human papillomavirus" was also suggested as a separate diagnostic statement.

The fact that there have been three systems, over a period of 30 years, for classification of a group of common and readily studied lesions suggests an incompletely understood but still developing concept of the underlying biology and its correlation with morphology. It may also reflect the practical difficulties of consistently and reliably dividing into two, three or four categories, what is perceived by many pathologists to be a continuum, using morphological criteria. However, there is no proof that this group of lesions is a continuum, in terms of either the genetic and viral changes that induce the lesions, or the probability of developing malignancy.

The evolutionary potential of intraepithelial lesions has been demonstrated by long term follow up studies. 50% of

patients with CIN 1 lesions developed CIN 3 lesions over 9 years, whilst 28% either progressed to grade 2 or remained at grade 1 (Richart and Barron, 1969). In animals, cervical lesions also develop through progressive stages of CIN 1 to CIN 3 and invasive carcinoma (Rubio and Lagerlof, 1974). There is variability in the progression rates, but the higher the grade the shorter is the transition time to CIN 3; regression also occurs but mostly in low grade lesions (Richart and Barron, 1969). There is evidence that strongly incriminates CIN 3 as a precursor of invasive cancer. Foci of CIN 3 can often be found in the same cervix as invasive carcinoma, usually at an adjacent site. Invasive carcinoma developed in 60% to 70% of women with carcinoma in situ/CIN 3, followed over the long term without treatment (Kottmeier, 1961; Boyes et al, 1963), although a more recent review of several studies attempting to define the natural history of carcinoma in situ/CIN 3 suggested that only 20-30% of lesions progress to invasive carcinoma within a period of 5-10 years (Chang, 1990). Most, but not all, new cases of carcinoma occur in a population of women with previously diagnosed CIN (Ferenczy and Winkler, 1987).

Progression to invasive cancer usually occurs at the squamocolumnar junction of the transformation zone - at the region of cervical ectopy (or ectropion). Previously, 80% - 90% of invasive carcinomas at this site were believed to be squamous cell carcinomas, with

adenocarcinomas accounting for most of the remainder. The relatively recent use of mucin stains (Alcian Blue/Periodic Acid Schiff or AB/PAS) has revealed that only about 70% of cervical cancers are purely squamous, and between 20% and 30% have to be reclassified as poorly differentiated adenocarcinomas or as mixed adenosquamous carcinomas (Yajima et al, 1984; Benda et al, 1985; Buckley et al, 1988). There are three major categories of cervical carcinomas, which have been redefined and resubclassified recently (Buckley and Fox, 1989).

(1) Squamous cell carcinomas are defined as neoplasms "showing evidence of squamous differentiation, eg keratinisation and/or intercellular bridge formation, and not showing any evidence of glandular differentiation or mucus secretion", and subdivided as well differentiated (large cell tumours showing well marked differentiation), moderately differentiated (large cell tumours showing focal keratinisation), and poorly differentiated (large or small cell tumours with minimal evidence of keratinisation). Verrucous and papillary squamous carcinomas are also separate entities.

(2) Adenocarcinomas can often be identified by their obvious glandular differentiation, but the poorly differentiated tumours may only be detected by demonstration of mucus secretion using mucin stains. Adenocarcinomas are defined as "neoplasms with no evidence of squamous differentiation, but showing either



formation of glandular structures or widespread mucus secretion, ie in at least 75% of the tumour cells". Most are recognisably of endocervical type, but a variety of others are described (endometrioid, papillary serous, clear cell, mesonephric, enteric) reflecting the capacity of cells of Mullerian origin to differentiate along alternative pathways, or undergo metaplastic change to enteric type.

(3) Mixed adenosquamous carcinomas, contain both squamous and adenocarcinomatous elements. These behave differently from true squamous and true adenocarcinomatous tumours. They were initially subdivided into "true" adenosquamous carcinomas, with one third or more of tumour cells secreting mucus, and "squamous carcinomas with mucus secretion", in which only a small proportion of tumour cells secrete mucus (mucoepidermoid carcinomas) (Buckley et al, 1988). They behave in a biologically identical manner, and the distinction is no longer justifiable (Buckley and Fox, 1989). Glassy cell carcinomas and adenoid cystic carcinomas are regarded as separate mixed cell entities. It is thought that mixed cell tumours arise from bipotential undifferentiated subcolumnar endocervical "reserve" cells, capable of differentiation to both squamous and glandular cells.

Only a small prortion of primary cervical carcinomas cannot be classified into these three groups, and include small cell carcinomas (neuroendocrine, basaloid, and

subcolumnar reserve cell), and undifferentiated carcinomas (Buckley and Fox, 1989). Metastatic carcinomas at this site are very rare.

The two most important features of cervical carcinomas of prognostic significance are the tumour size/extent of local disease (local staging) and lymph node metastasis, although there is considerable variability of outcome within individual stages (Prempreet et al, 1985; Inoue et al, 1986; van Brommel et al, 1987). Of the histological features, cell type, cytological grade and lymphatic/vascular permeation all influence outcome. Adenocarcinomas have poorer prognoses than squamous cell carcinomas (Van Nagell et al, 1979; Moberg et al, 1986), and mixed carcinomas have the highest levels of association with nodal metastasis in early stage, particularly the poorly differentiated adenosquamous carcinomas detectable only with mucin stains (Ireland et al, 1987; Buckley et al, 1988). Cytological grading of cervical squamous carcinomas is of little prognostic value, as the nodal metastasis rate is identical for well, moderately and poorly differentiated tumours, with the exception of non-keratinising small cell squamous carcinomas which are more likely to give rise to lymph node metastases (Van Nagell et al, 1979; Buckley et al, 1988). Poorly differentiated adenocarcinomas have a lower survival rate than well differentiated adenocarcinomas (Prempreet et al, 1985; Weiss and Lucas, 1986). Lymphatic/vascular permeation by cancer cells correlates

well with nodal metastases, and thus with poor prognosis (Buckley et al, 1988; Buckley and Fox, 1989).

#### 2.1.2 HUMAN PAPILLOMAVIRUSES IN CERVICAL NEOPLASIA

HPV infections have been categorised as clinical, subclinical and latent (Schneider, 1990). Clinical HPV infections, such as condylomas, either cause symptoms to the patient, or are visible to the naked eye. Subclinical HPV infections, such as intraepithelial neoplasia, may not cause symptoms, but produce histologically identifiable abnormalities. In contrast, latent HPV infections can not be diagnosed clinically or histologically, as the presence of the virus does not cause any clinically or morphologically apparent abnormalities. Clinical infection is often associated with virion particle production, but it is not known whether subclinical or latent infections are productive or non-productive states. The majority of investigations of subclinical and latent infections identified only HPV DNA, and did not test for virion particles within the lesions. Even in productive HPV infections the number of virion particles is usually very low. Analysis of the presence of koilocytes or dyskeratocytes in cervical smears in routine screening programmes, detects a prevalence of 0.7 - 3% (De Brux et al, 1983; Meisels and Morin, 1986), whereas, of the cervical smears of STD clinic attendants, 8 - 13% show these classical cytological markers of HPV infection (Drake et al, 1987).

The prevalence of HPV, by detection of viral DNA, in the normal population is controversial. Prevalence figures of 0% to 84% detected by PCR have been reported (Manos et al, 1990; Schneider, 1990). Some studies using DNA hybridisation detected HPV in 10% - 12% of subjects (Toon et al, 1986; deVilliers et al, 1987). Varying assay sensitivities and target populations probably account for the wide disparity of results. Since in the majority of studies not all patients were colposcopically and histologically examined for subclinical HPV infection, some of the HPV positive patients may have been subclinically infected and some latently infected with HPV. A consensus figure, derived from published studies, of approximately 10% of women with HPV DNA positive but morphologically non-neoplastic cervixes seems reasonable, and this would provide an enormous pool for propagation of HPV within the population. Transmission of HPV appears to take place via the sexual and peripartal routes, and there is evidence that immunosuppression and pregnancy are risk factors for the acquisition or reactivation of latent or subclinical HPV infection (Schneider, 1990). However, many studies have used non-random, and possibly non-normal control samples derived from patients attending clinics, including those for sexually transmitted diseases and other gynaecological diseases. It is clear that accurate and reliable data for HPV prevalence in a truly normal population are lacking.

Broadly similar patterns of prevalence of HPV types in cervical neoplasms have been found by many workers, but some variation has been observed and attributed to geographical differences, focal heterogeneity of HPV replication within lesions sampled, or variability in the sensitivity of the assays employed. In genital warts and low grade cervical intraepithelial neoplasia (CIN 1) the commonest HPV types are 6b and 11, whereas HPV types 16 and 18 have been detected in association with up to 80% of high grade lesions (CIN 2 and CIN 3), and with up to 100% of invasive cervical cancers (Gissmann, 1984; Brescia et al, 1986; Syrjanen, 1986; Pater et al, 1986; Pfister, 1987b; deVilliers et al, 1987; Xiao et al, 1988; Stanley, 1990).

There is a progressive increase in positivity of HPV 16 or 18 in 4 groups of genital lesions: condylomas, all grades of dysplasia, carcinoma in situ, and invasive cancers (of cervix, vulva and penis); and a complementary decrease in HPV types 6b and 11 in these 4 groups, permitting categorization of HPVs into high risk or low risk types (Gissmann, 1984).

Amongst the high risk types, HPV 18 has been suggested to be a more aggressive type than HPV 16, because it is associated with (1) more glandular cancers, known to have a poorer prognosis, in contrast to the predominance of HPV 16 in squamous carcinomas (Wilczynski et al,

1988), (2) more high grade tumours (Barnes et al, 1988), and (3) a more rapid transition from CIN to cancer, at least as indicated by the CANCER/CIN prevalence ratio. HPV 18 has been associated with a higher CANCER/CIN ratio of 7.3 (22%/3%), compared to 1.1 for HPV 16 (41%/37%) (Kurman et al, 1988).

Of the greater than 60 HPV types, many others infect the cervix. Those HPVs associated only with benign lesions include types 30, 40, 42, 43, 44 and 58. HPVs found in either CIN or cancers include 31, 33, 35, 39, 45, 51 and 52 (Lorincz et al, 1986, 1987a and 1987b; Beaudenon et al, 1986 and 1987; Naghashfar et al, 1987; deVilliers, 1989). Insufficient clinical information has accumulated to authoritatively assign levels of clinical risk or associated tumour aggression to these additional HPV types. However, in vitro transfection of DNA from HPV types 16, 18, 31 and 33 (but not 6b and 11) induced immortalization and aneuploidy in normal human genital keratinocytes, and transformation of primary rodent cells with activated ras, suggesting a high oncogenic potential for all these types (Storey et al, 1988; Crook et al, 1988; Woodworth et al, 1989).

Nonetheless, a small proportion of cervical cancers appear to remain negative for HPV DNA. Compared with HPV positive carcinomas, these have a poorer prognosis in terms of distant metastases and relapse, and may form a biologically distinct subset of tumours (Riou et al,

1990). It has not been possible in the past to determine whether HPV is absent from these, present at copy numbers below the level of detection, or represented by some as yet uncharacterised HPV types. Some of these problems may be overcome by use of the polymerase chain reaction (PCR), which can detect a single target DNA sequence amongst a million diploid cells (Saiki et al, 1988). This technology has been used to identify as few as 1-2 copies of an HPV genome integrated into a cervical carcinoma cell line (SiHa), in a sample of only 10 cells (Shibata et al, 1988a). Using PCR-based methods without optimisation of discrimination between HPV types, viral DNA has been detected in cervical smears from women with no history of cervical lesions attending routine screening clinics (Melchers et al, 1989; Young et al, 1989), and in substantial proportions of women with CIN or cervical carcinomas (Melchers et al, 1989; Young et al, 1989; Shibata et al, 1988a and 1988b; Kiyabu et al, 1989). The detection rate often exceeded that of non-PCR methods (Toon et al, 1986; deVilliers et al, 1987).

To define more clearly the levels of association of HPV with cervical neoplasia, a new assay was designed and optimised for HPV detection. This used PCR to amplify conserved DNA sequences within the E6 genes of 5 common genital HPV types. The assay differed from previously used PCR methods in several ways. Distinction between HPV types was maximised by use of primers uniquely competent

to amplify each virus type. The amplified DNA was identified by hybridisation with HPV type specific oligonucleotide probes, which were visualised by non-radioactive means. Stringent precautions were included to avoid false positives secondary to sample contamination.



## 2.2 MATERIALS AND METHODS

### 2.2.1 MATERIALS

Paraffin sections were taken from 1-2 yr old archival blocks of formaldehyde-fixed cervical squamous carcinomas. Frozen tissue was obtained from a colposcopy clinic to which patients were referred as a result of abnormal smears. HeLa and SiHa cervical carcinoma cell lines and Raji lymphoblastoid cells were obtained from the American Type Culture Collection. Oligonucleotide primers and probes were synthesised on an Oswel Gene Synthesiser (Dept. of Chemistry, University of Edinburgh, UK) and were HPLC purified. Probes were biotinylated during synthesis, by direct addition of a biotin moiety, on a 15 carbon atom linker arm, to the 5' end of the oligonucleotide probe. A 1 kilobase ladder marker (Gibco-BRL) was used as the agarose gel electrophoresis standard. HPV DNA sequences, derived from the EMBL genetic sequence database, were analysed and oligonucleotide sequences selected using the University of Wisconsin (UW) Genetics Computer Group Software (Cameron, 1988; Devereux et al, 1984).

### 2.2.2 PARAFFIN SECTIONS

One 20um section was cut from paraffin-wax embedded tissue blocks taken from uteri resected for cervical carcinoma, and placed in a 1.5ml Eppendorf tube. Template DNA was prepared by digestion with 100ul of 0.06mg/ml

proteinase K in 10mM Tris, 0.1mM EDTA (pH8.0) at 48°C for 5 days (Jackson et al, 1990a and 1990b). Following centrifugation at 100g for 5 min, the supernatant was twice extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in 0.5 vol 7.5M ammonium acetate and 2.5 vol ethanol at -20°C for 16 hours. After centrifugation at 100g for 15 min, the resulting DNA pellet was resuspended at 20°C over 48 hours in 100ul 10mM Tris, 0.1mM EDTA (pH8.0) (TE buffer). To 10ul of this resuspended DNA was added 90ul of PCR solution, and this was heated to 98°C for 10 min prior to amplification.

#### 2.2.3 FROZEN SECTIONS

Two 20um sections were cut from frozen tissue blocks of punch biopsies of cervical intraepithelial neoplasia. Prior to thawing, the frozen sections were placed on the surface of frozen distilled water in a 1.5ml Eppendorf tube. Concentrated PCR solution was added to give a final volume of 100ul and this was heated to 98°C for 10 min prior to amplification.

#### 2.2.4 CULTURED CELLS

Up to  $10^4$  cultured cells, suspended in 25ul volume, were prepared for PCR either by boiling in PCR buffer for 10 min (cell lysis procedure 1), or by lysis at 55°C for 60 min in PCR buffer with 0.45% NP40, 0.45% Tween and

0.06mg/ml proteinase K, followed by incubation at 98°C for 10 min to inactivate the proteinase K enzyme (cell lysis procedure 2). A further 75ul of PCR solution was added prior to amplification.

#### 2.2.5 POLYMERASE CHAIN REACTION

The PCR solution consisted of PCR buffer (50mM KCl, 10mM Tris-HCl (pH8.3 at room temperature), 1.5mM MgCl<sub>2</sub>, 0.01% gelatin), 200uM of each dNTP, 1.0uM of each primer, 2.5U *Taq Polymerase* or *Amplitaq* (Perkin-Elmer-Cetus) and template (tissue section, cell suspension or DNA solution) in a total volume of 100ul overlaid with 100ul mineral oil. Samples were subjected to 30 cycles of PCR on an automated heating block (Hybaid), each cycle consisting of DNA duplex denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and DNA synthesis by primer extension at 72°C for 3 min. During the final cycle, the extension step lasted 10 min. 20ul of the reaction product was electrophoresed on a 2% agarose (3:1 Nusieve GTG:Seakem) gel containing 2ug/ml ethidium bromide and visualised under UV light. Amplified DNA within the remaining 80ul reaction product was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in 0.5 vol 7.5M ammonium acetate and 2.5 vol ethanol. The resulting DNA pellet was resuspended in 20ul 10mM Tris, 0.1mM EDTA (pH8.0) (TE buffer).

Stringent precautions were taken to minimise the risk of contamination of the PCR solution with HPV DNA from unwanted sources (Kwok and Higuchi, 1989). Tissue sections from different cases were cut using either separate microtome blades or previously unused regions of the same blade (when taking sections from small punch biopsies). Sections cut from a block of tissue not containing HPV (eg myocardium) were included when appropriate, to investigate the possibility of transfer of HPV DNA from case to case, during section cutting and dewaxing. To reduce the chance of carryover of amplified product three separate laboratories were utilised for (1) preparation of the PCR solution, (2) amplification in the automated heating block, and (3) analysis of the amplified DNA by gel electrophoresis or dot blot hybridisation. The components of the PCR solution were prepared in small aliquots prior to use, and were added to the reaction in a laminar air flow class II biological safety cabinet, using positive displacement pipettes exclusively dedicated to this purpose. Throughout this the operator adopted a microbiological standard sterile technique and wore disposable gloves. The PCR solutions were added to the template solutions or sections in another hood. Template solutions were transferred using a separate set of dedicated pipettes, which were immersed in 0.1M hydrochloric acid for 10 min and rinsed in autoclaved distilled water prior to use. A template-free negative control, exposed to the same preparative

environment, was included in every assay in order to detect contamination should it occur, as was a positive control containing 5ng of the appropriate plasmid with cloned HPV DNA to confirm successful PCRs.

#### 2.2.6 DOT BLOT HYBRIDISATION

Nitrocellulose filters were pretreated with distilled water followed by 15 x SSC (standard sodium citrate). The amplified DNA was resuspended in 20ul TE buffer and serially diluted ten- and one hundred-fold in 15 x SSC. 10ul of the original and the diluted samples, made up to a final volume of 100ul in 15 x SSC, were subjected to heat denaturation at 98°C for 5 min, and vacuum blotted on to nitrocellulose filters, using "Hybridot" manifold (Gibco-BRL). The sample wells were washed through with a further 100ul 15 x SSC.

The filters were baked at 80°C for 2h in a vacuum oven and prehybridised for 1 h at 42°C in 5 x SSC, 25mM  $\text{NaH}_2\text{PO}_4$  (pH6.5), 5 x Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS) and 200ug/ml denatured salmon sperm DNA. The prehybridisation solution was replaced with the hybridisation solution which contained the same components, with the addition of 0.8uM biotinylated oligonucleotide probe (heated to 98°C for 10 min before use to denature any secondary structure), and differing concentrations of deionised formamide optimised for the different probes: 10% for HPV 11, 15% for HPV 16, 30% for

HPV 18 and 0% for HPV 6b and 33 (Albretsen et al, 1988). Filters were hybridised at 42°C for 16-20h, washed twice at 20°C in 2 x SSC with 0.1% SDS, twice at 42°C in 0.5 x SSC with 0.1% SDS and baked at 80°C for 1h in a vacuum oven.

Bound, biotinylated probe was detected using a modification of the "Blugene" protocol (Gibco-BRL). Non-specific streptavidin binding sites were blocked by incubating the filters for 20 min at 42°C in a solution of 0.1M Tris-HCl (pH7.5), 0.1M NaCl, 2mM MgCl<sub>2</sub>, 0.05% Triton X-100 (buffer 1) with bovine serum albumin added to a final concentration of 3% (buffer 2). The filters were baked under vacuum at 80°C for 1h, rehydrated in buffer 2 for 10 min, incubated at 20°C for 10 min in 2ug/ml streptavidin solution (Gibco-BRL), washed twice in buffer 1, and incubated at 20°C in 1ug/ml biotinylated alkaline phosphatase solution (Gibco-BRL) for 10 min. The filters were then washed twice in buffer 1 at 20°C for 10 min and twice in 0.1M Tris-HCl (pH9.5), 0.1M NaCl, 50mM MgCl<sub>2</sub> (buffer 3) at 20°C for 10 min. Bound alkaline phosphatase was visualised by incubation in the dark, at 20°C in a solution of 0.33mg/ml nitro-blue tetrazolium (NBT) and 0.166mg/ml 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) in buffer 3. After 1-2 h, deposition of the blue-purple coloured reaction product was terminated by immersing the filters in 20mM Tris (pH7.5), 5mM EDTA and the filters baked under vacuum at 80°C for 1-2 min.

### 2.2.7 RESTRICTION ENZYME MAPPING

Amplified DNA products, resuspended in 20ul restriction enzyme buffer, were digested at 37°C for 16-20h. 10ul DNA solutions of both HPV 6b and 11 were restricted with DdeI (22 units) (Sigma), HPV 16 and 18 with HinfI (20 units) (Sigma) and HPV 33 with DraI (24 units) (Amersham). The digested DNA was subjected to electrophoresis on a 2.5% agarose gel containing 2ug/ml ethidium bromide and visualised under UV light.

## 2.3 DESIGN AND OPTIMISATION OF POLYMERASE CHAIN REACTION FOR HPV DNA DETECTION

A type-specific and highly sensitive, non-radioactive assay was designed for detection of HPV DNA in tissues. DNA sequences within the conserved HPV early gene E6 were selected by computer search to optimise distinction between HPV types. These were amplified using the polymerase chain reaction (PCR), with different primer pairs uniquely and exclusively effective for each of HPV types 6b, 11, 16, 18 and 33. The amplified DNA products were identified by both non-radioactive oligonucleotide hybridisation and restriction endonuclease mapping. This technique was successfully applied to HPV-containing plasmid DNA, cultured cells, DNA extracted from genital warts, and frozen and archival paraffin-embedded sections of cervical intraepithelial neoplasia and carcinoma. The assay clearly distinguished HPV types with close sequence homology and was sufficiently sensitive to detect HPV DNA in 3-5 SiHa cells (each containing 1-2 copies of integrated HPV 16 DNA), amongst ten thousand non-HPV containing cells.

### 2.3.1 SELECTION OF HPV TARGET SEQUENCES FOR AMPLIFICATION

HPV sequences suitable for amplification, to provide diagnostic, type-specific products, must fulfil several criteria. They must (1) remain intact following viral DNA



integration, (2) have a high degree of conservation between viruses of the same HPV type, such as genes encoding transforming proteins, (3) show type-specific sequence divergence, (4) be flanked by sequences suitable for use as efficient PCR primers, and (5) contain a central type-specific sequence appropriate for oligoprobe hybridisation. The E6 and E7 genes fulfil criteria 1 and 2, and were therefore searched for suitable target sequences which meet criteria 3 to 5 and are of 100-300 bp in length.

To distinguish between the most similar viral types, the E6 gene sequence of HPV 6b was compared with that of HPV 11 (these two viruses have an overall nucleotide homology of 82% - the highest of any 2 genital HPV types), using the University of Wisconsin (UW) computer program COMPARE. Similarly, the E6 sequence of HPV 16 was compared with those of types 18 and 33. The sequence homologies were displayed graphically as dotplots using the UW program DOTPLOT (figure 2.1). The DNA regions with dissimilar sequences appeared as gaps in the diagonal line of colinear homology in the dotplots. These were then studied at the nucleotide level, using the UW program GAP, which produced optimal sequence alignment with the most similar HPV type, allowing determination of the exact extent of base mismatching.

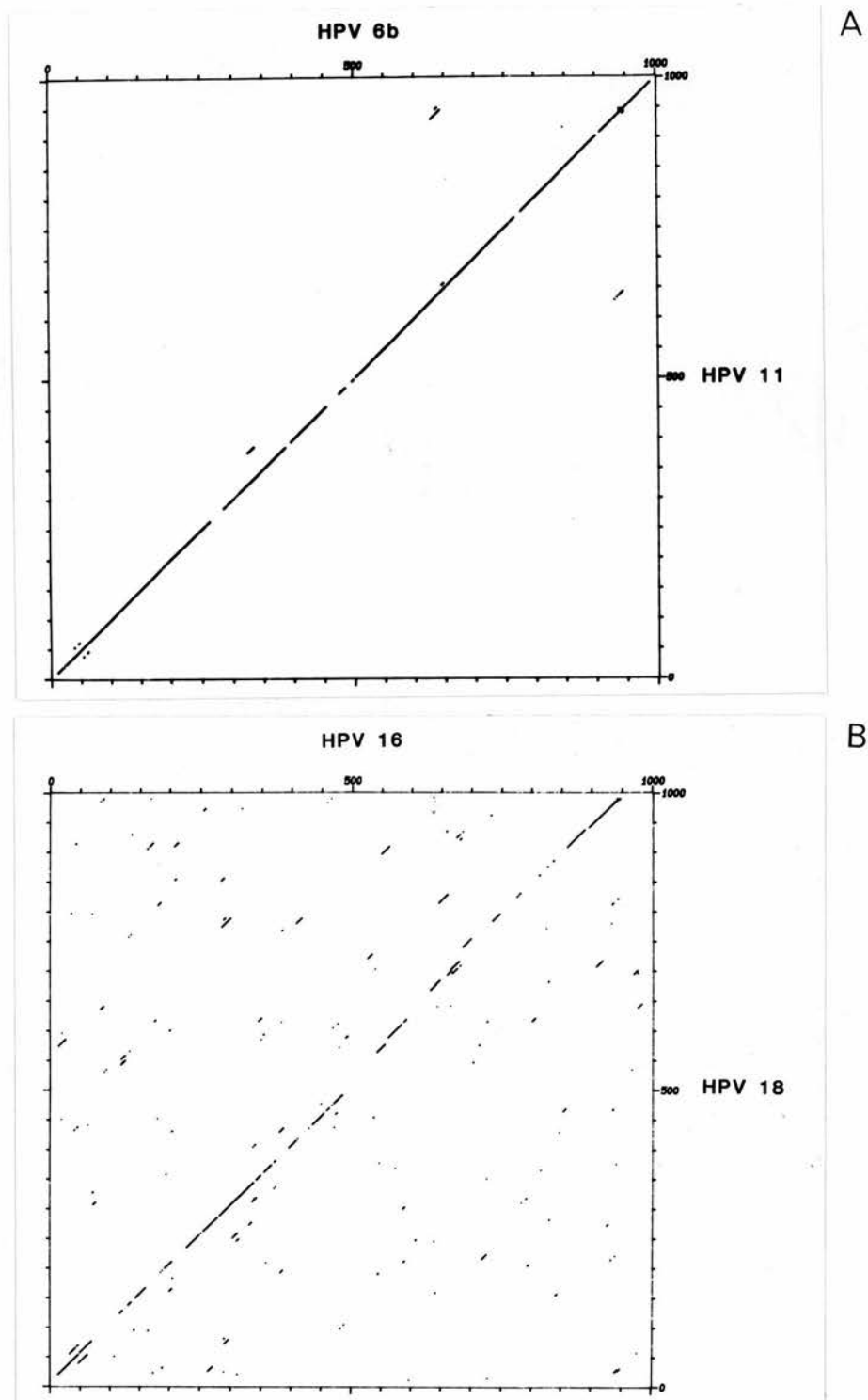


Figure 2.1

Computer generated dotplots of the comparison of homologies of the first 1000 nucleotides (which contain the E6 and E7 sequences) of (A) HPV 6b with HPV 11, using a window of 20 bases in length, and a stringency of 15, with each dot plotted representing the occurrence of 15 or more identical bases out of 20 being compared at that position; and of (B) HPV 16 with HPV 18, using a stringency of 13 within a window of 20 bases. The high density of dots along the diagonals illustrates the strong colinear homologies, and the gaps indicate the local regions of relative nucleotide mismatching, which are appropriate for use as type-specific PCR primer or probe sequences.

TABLE 2.1

## OLIGONUCLEOTIDES

HPV	PRIMER/ PROBE	SEQUENCE	POSITION	HOMOLOGY	COMPARATOR
6b	Primer 1	CCT GTT TCG AGG CGG CTA TCC ATA	260-283	58	11
6b	Primer 2	GTA CAA TTT AGC TTT ATG AAC CGC GCC TTG GTT	464-496	64	11
6b	Probe	TAA ACA AGA CAT CTT AGA CGT GCT AAT TCG GTG C	380-413	71	11
11	Primer 1	TGT GTG GCG AGA CAA CTT TCC CTT	260-283	58	6b
11	Primer 2	TGG TTA TTT AGT TTT ATG AAG CGT GCC TTT CCC	464-496	64	6b
11	Probe	CAA TGA AGA TAT TTT AAA AGT GTT AAT TCG TTG T	380-413	71	6b
16	Primer 1	CTG CAA GCA ACA GGT ACT GCG ACG	199-222	42	18
16	Primer 2	CAT ACA TCG ACC GGT CCA CC	495-514	35	18
16	Probe	TAT TAA CTG TCA AAA GCC ACT GTG TCC TGA	412-441	65	18
18	Primer 1	AAA CTA ACT AAC ACT GGG TTA TAC A	378-402	44	16
18	Primer 2	ATG GCA CTG GCC TCT ATA GT	502-521	35	16
18	Probe	CCT GCG GTG CCA GAA ACC GTT GAA TCC AGC	419-448	65	16
33	Primer 1	AAC AGT TAA AAA ACC TTT AAA	378-398	48	16
33	Primer 2	AGT TTC TCT ACG TCG GGA CCT C	528-549	41	16
33	Probe	AAA ACG ACA TGT GGA TTT AAA CAA ACG ATT T	453-483	50	16

**Note:** Nucleotide sequences (5' to 3') of the pairs of PCR primers and oligoprobes for each HPV type, their positions within the viral genome according to the EMBL database nomenclature, and the percentage homology (column 5) with the equivalent DNA sequence of the most closely matching alternative HPV genome (column 6).

The target regions that were selected contained three sequences with a high degree of base mismatching - one at either end for PCR primer 1 (P1) and primer 2 (P2) sequences and one central sequence for the oligoprobe (Pr) (table 2.1). These sequences had such low levels of homology with other HPV types that no significant binding would be expected to occur at annealing or hybridisation temperatures of 40-60°C. To improve the sequence specificity of the probes, oligonucleotides of up to 33 bases in length were selected, so permitting the use of high stringency washing conditions. Occurrences of the primer and probe sequences (including up to 5 mismatches), elsewhere in any of the HPV types were excluded by computer search using the UW program FIND.

Guidelines for primer selection, suggested by Saiki (1989), were taken into account, including GC content, predicted secondary structure and 3' complementarity. The GC content of the primers varied from 39% to 60% with the exceptions of HPV 18 primer 1 (32%) and HPV 33 primer 1 (19%). The possible formation of significant secondary structures by the single stranded DNA primers, which might interfere with primer annealing during PCR, was assessed using the UW programs FOLD and SQUIGGLES. Secondary structures, known as stem loops, with 5 or fewer internal base pairings were predicted for all of the primers. No significant complementarity between the 3' ends of paired primers (for each HPV type) was identified, thus limiting the risk of primer dimer

formation.

### 2.3.2 TARGET DNA AMPLIFICATION

The protocol for amplification of target HPV DNA by the polymerase chain reaction was optimised using plasmid DNA as template, containing cloned full HPV genomes, and a primer annealing temperature of 42°C. Amplified sequences were visualised as single bands by agarose gel electrophoresis, and were of the predicted lengths. Amplified fragments of HPV 6b and HPV 11 were 237 base pairs (bp) in length, while those of HPV 16, 18 and 33 were 316, 144 and 172 bp respectively (figure 2.2). Successful amplification of HPV DNA by this technique was achieved using a variety of templates, including DNA extracted from a fresh vulval wart, and from frozen and paraffin wax sections of the same lesion (figure 2.2). The primer annealing temperature ( $T_a$ ) was raised to 55°C for assays of tissues, although a  $T_a$  of up to 60°C has been successfully employed. This increases the specificity of binding of primers to target HPV DNA and reduces the risk of primers binding to near-complementary sequences in the host cell genome with ghost band production.

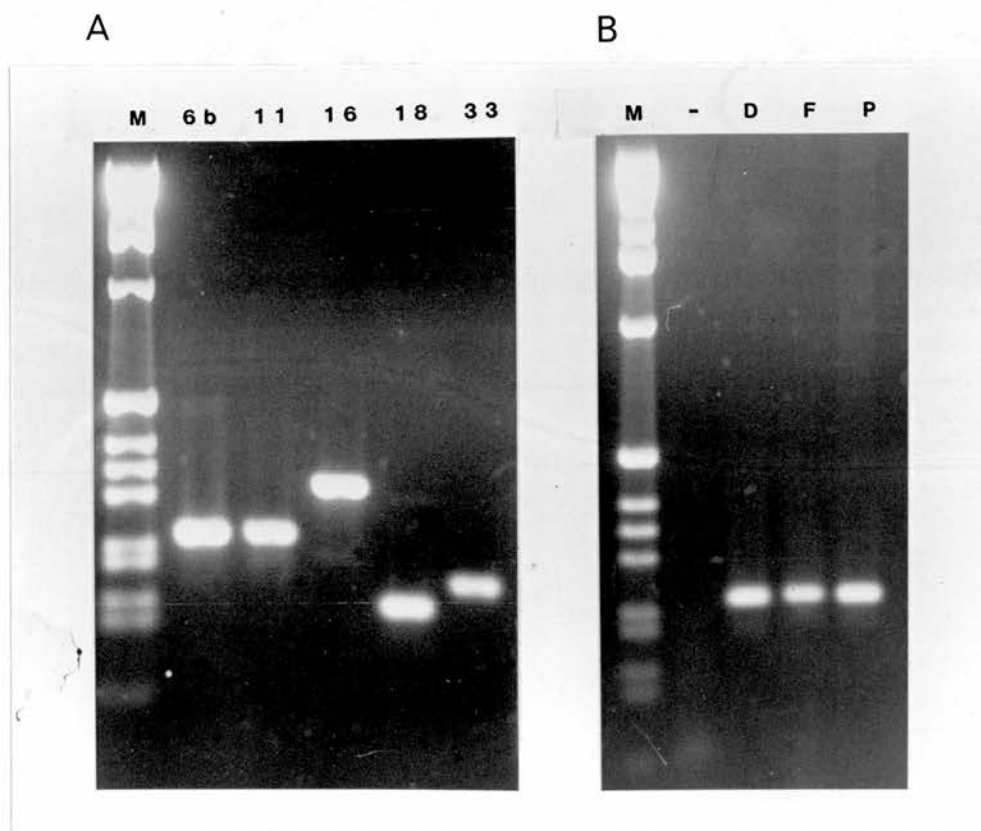


Figure 2.2

Agarose gel electrophoresis of amplified HPV DNA: (A) using type-specific pairs of primers with cloned DNA for types (a) HPV 6b (fragment length 237 bp), (b) HPV 11 (237 bp), (c) HPV 16 (316 bp), (d) HPV 18 (144 bp) and (e) HPV 33 (172 bp); and (B) using HPV 11 primers with samples of a vulval wart, harbouring HPV 11, in the form of extracted DNA (D), frozen sections (F), or paraffin embedded sections (P); a "template-free" negative control track (-), and 1 kb ladder marker tracks (M) are included.

### 2.3.3 IDENTIFICATION OF AMPLIFIED DNA

Two methods were employed to confirm that the amplified DNA, as visualised by gel electrophoresis, contained the appropriate HPV target sequence. Dot blots of amplified DNA were hybridised with oligoprobes, each specific for a single HPV type. The biotinylated oligoprobes were visualised by formation of a complex with streptavidin and biotinylated alkaline phosphatase followed by deposition of a coloured reaction product using NBT and BCIP (figure 2.3). Alternatively, the amplified DNA was analysed by restriction endonuclease mapping, using enzymes which cut a maximum of twice within the target sequences and generated diagnostic fragments from each of the 5 HPV types. The sizes of the cleaved DNA fragments were determined by gel electrophoresis (figure 2.4) and were found to be of predicted lengths. Thus, amplified products of HPV 6b, digested with DdeI, produced two fragments of 133 and 104 bp; HPV 11 also cleaved by DdeI, produced two bands of 194 and 43 bp. HinfI digestion of amplified DNA of HPV 16 gave 200, 76 and 40 bp bands, and of HPV 18 gave 81 and 63 bp bands. DraI restriction of HPV 33 amplified DNA gave fragments of 79, 75 and 18 bp.

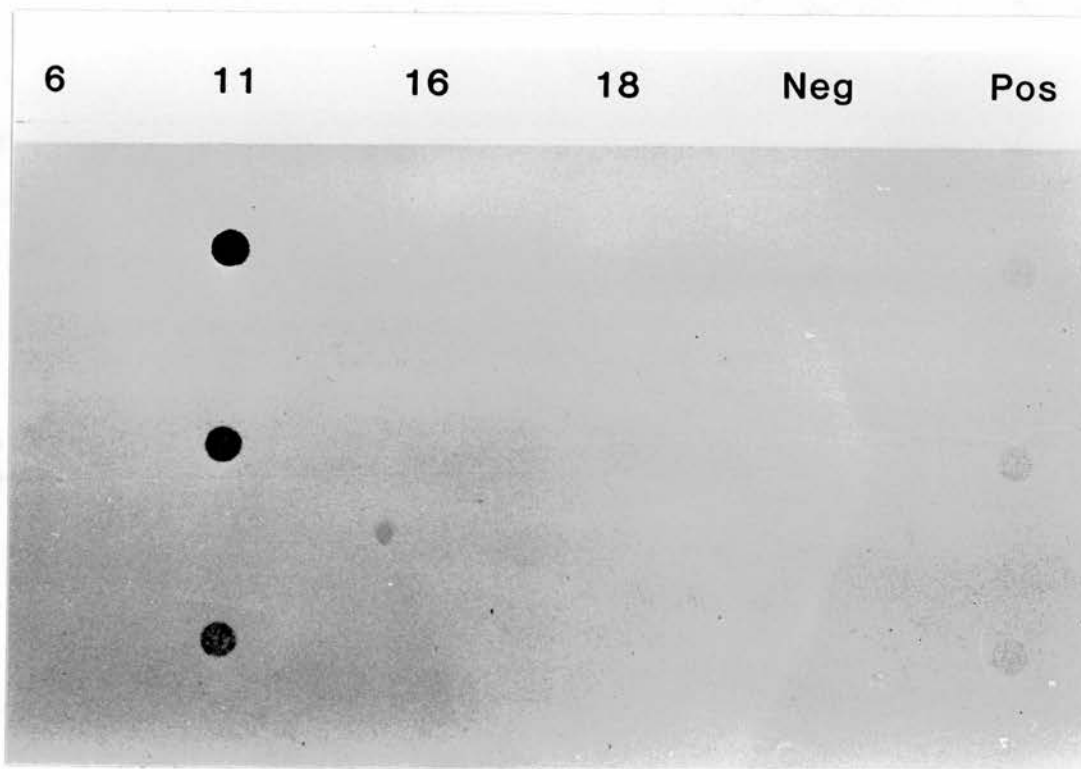


Figure 2.3

Dot blot showing HPV type-specificity of oligoprobe hybridisation to amplified DNA. DNA was immobilised onto the nitrocellulose filter undiluted and in ten- and one hundred-fold dilutions, using PCR generated amplified DNA from HPV types 6b, 11, 16 and 18, and plasmid DNA of pBR322 (Neg) and of pHPV11 (Pos), acting as negative and positive controls respectively. The filter was hybridised with the biotinylated oligoprobe for HPV 11, which was detected by a streptavidin and alkaline phosphatase reaction. The probe identifies only amplified DNA of the same HPV type.



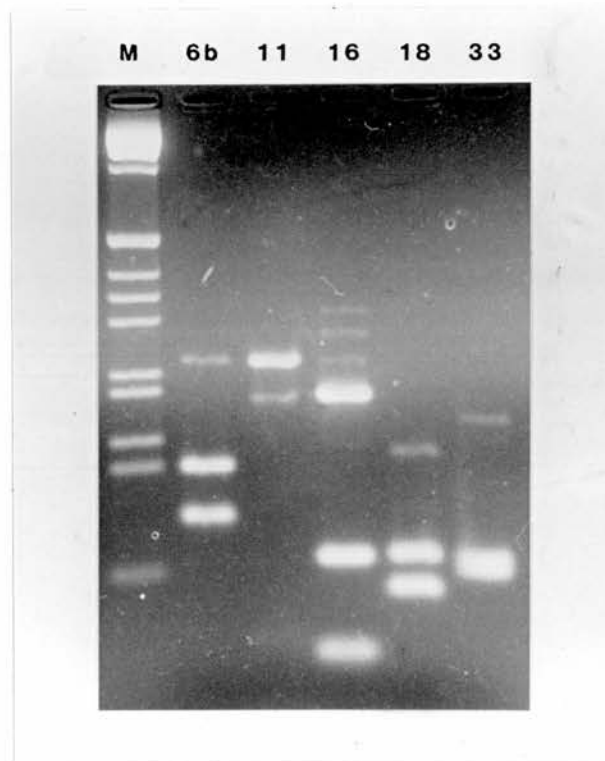


Figure 2.4

Amplified DNA from each of the HPV types was identified by restriction endonuclease mapping, with the sizes of digested DNA fragments determined by agarose gel electrophoresis. A unique pattern of DNA fragments was observed for each HPV type/enzyme combination: (a) HPV 6b/Dde I gave 133 and 104 bp fragments; (b) HPV 11/Dde I gave 194 and 43 bp fragments; (c) HPV 16/Hin fI gave 200, 76 and 40 bp fragments; (d) HPV 18/Hin fI gave 81 and 63 bp fragments; and (e) HPV 33/Dra I gave 79 and 75bp fragments, the remaining 18 bp fragment is not visible in this gel. In some reactions small quantities of undigested amplified DNA products are visible. A 1kb ladder marker track (M) is shown.

#### 2.3.4 HPV TYPE SPECIFICITY OF AMPLIFICATION AND HYBRIDISATION

To establish type specificity of primer-directed amplification each set of primers was tested with template plasmid DNA of the 5 HPV types 6b, 11, 16, 18 and 33. Amplified DNA fragments, detected by gel electrophoresis, were generated only when the primer pairs were used with template DNA of the same viral type; no amplification occurred when primers of one type were applied to template DNA of another (figure 2.5). To establish type specificity of oligoprobe hybridisation, amplified target DNA sequences from all 5 HPV types were dot blotted onto nitrocellulose filters and tested for cross-hybridisation using the 5 HPV type-specific oligoprobes. Each oligoprobe generated a positive hybridisation signal only with amplified target DNA of the same HPV type (figure 2.3).

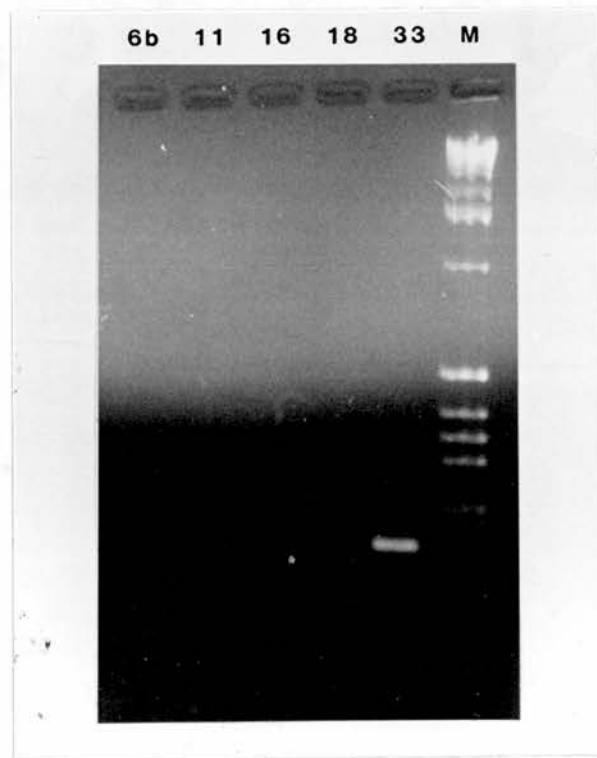


Figure 2.5

The specificity of each pair of primers to direct amplification by PCR of a single HPV type was tested using cloned HPV genomes as templates. Agarose gel electrophoresis shows the products of PCRs using HPV 33 primers with template plasmid DNA of HPV types 6b, 11, 16, 18 and 33. A 1kb ladder marker track (M) is included. Positive amplification is achieved only with HPV 33 template.

### 2.3.5 SENSITIVITY OF THE POLYMERASE CHAIN REACTION

The ability of the assay to detect HPV DNA in low abundance was determined using as template, cultured cervical carcinoma-derived cell lines with known HPV DNA content. Test cell samples consisted of serial dilutions of HeLa cells (containing 30-40 copies of HPV 18 per cell) and SiHa cells (containing 1-2 copies of HPV 16 per cell), mixed with  $10^4$  control Raji lymphoblastoid cells (without any HPV). Using appropriate oligonucleotide primers for 35 cycles of the PCR, amplified target DNA sequences were consistently detected from reactions containing five HeLa cells (data not shown), or five SiHa cells (figure 2.6). Quadruplicate reactions, nominally containing 4, 3, 2 or 1 SiHa cell were also analysed. Two out of four reactions containing three or four cells were positive, and none of the reactions containing one or two cells (figure 2.6). Since the actual number of cells in these very dilute suspensions is determined by the Poisson distribution, these results demonstrate a true threshold sensitivity of between 3 and 5 SiHa cells (3-10 integrated HPV DNA copies).

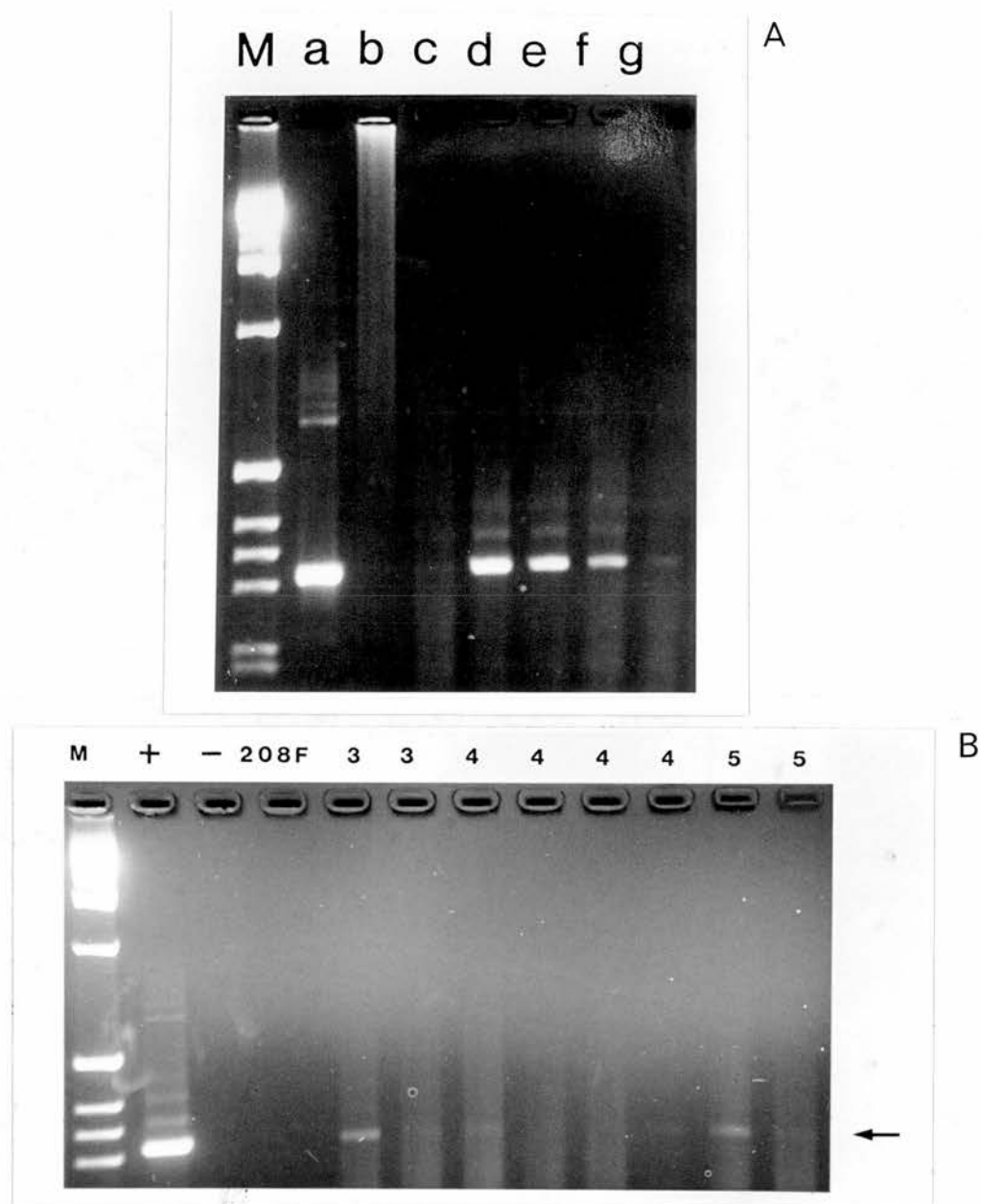


Figure 2.6

Sensitivity of the assay is shown by agarose gel electrophoresis of ethanol precipitated DNA from 80ul reaction solutions after 35 cycles of PCR, demonstrating amplification of viral DNA from SiHa cells (known to contain 1-2 HPV genomes) directed by primers to HPV 16. (A) The SiHa cells were serially diluted amongst a background of  $10^4$  Raji cells: (M) 1kb ladder marker track; (a) positive control pHPV 16 plasmid DNA; (b) negative control "template-free" reaction; (c) no SiHa cells; (d) 1000 SiHa cells; (e) 100 SiHa cells; (f) 10 SiHa cells (g) 5 SiHa cells. Positive amplification is seen in all reactions containing SiHa cells.

(B) Quadruplicate samples were analysed, each containing 3 or 4 or 5 SiHa cells on a background of 10,000 208F cells. The gel shows a positive pHPV16 plasmid control (+), a negative template free control (-), 208F cells without SiHa cells, two samples containing 3 SiHa cells, both of which are positive, 4 samples containing 4 SiHa cells, the first and fourth of which are positive, the second and third negative, and 2 samples of 5 SiHa cells both of which are positive. A 1 kb ladder marker track (M) is included.

### 2.3.6 DISCUSSION

An assay using PCR for detection of HPV DNA has been designed with novel features of optimised specificity and sensitivity, and this has been successfully applied to clinical specimens containing few cells. Absolute HPV type specificity was achieved by using two independent stages of oligonucleotide annealing. First, DNA from each HPV type was amplified using separate pairs of PCR primers with unique sequences. The large sizes of primer oligonucleotide, 20-33 bp, allow annealing temperatures of 50-60°C to be used during PCR, producing high stringency, and a reduced probability of illegitimate primer annealing to near-homologous genomic sequences. The paucity of ghost bands indicates a highly efficient amplification of legitimate target sequences. Second, identification of amplified DNA by hybridisation with type-specific oligoprobes confers a further degree of specificity. These oligoprobes were biotinylated during chemical synthesis, a further novel feature of this assay, thus the hazards associated with the use of radioactivity are avoided, and this efficient method of labelling provides a supply of oligoprobes which are ready to use. Other PCR-based HPV DNA detection assays have been described, using common primers that bind to the DNA of more than one HPV type (Shibata et al, 1988a; Snijders et al, 1990), or one stage procedures without identification of amplified DNA (Young et al, 1989). These are prone

to the theoretical risk - avoided in the present method - of incorrect identification of HPV type. The different sizes of amplified target DNA allow the combination of more than one pair of PCR primers in a single reaction (multiplex PCR). Thus, it has been possible to simultaneously analyse cervical neoplastic lesions for HPV types 6b and 16 and also for HPV types 11 and 18. The design logic of this method would also be generally applicable to HPV infections at other sites where detection of specific types is important, such as skin, where HPV types 5 and 8 have been associated with malignant lesions in renal allograft recipients and epidermodysplasia verruciformis patients (Arends et al, 1990).

The high sensitivity of HPV DNA detection was demonstrated by the detection, within a background of 10000 control non-HPV containing cells, of between 3-5 SiHa cells known to contain 1-2 copies of HPV 16. This was achieved using 30-35 cycles of PCR, which produces the maximum quantity of amplified target DNA limited by the "PCR plateau" effect (Saiki, 1989).

Highly sensitive PCR amplification has the advantage of applicability to small tissue samples, such as colposcopic target biopsies of the relatively small, circumscribed cervical acetowhite lesions typical of CIN. There is difficulty in identifying HPV by other methods in specimens with few cells, from which insufficient DNA

may be extracted. Presumably, this has contributed to the controversy over HPV involvement in these lesions. PCR is known to be more sensitive than Southern blotting and filter hybridisation in the detection of HPV in cervical scrapes (Melchers et al, 1989; Schiffman et al, 1991). PCR amplification of short sequences within the E6 gene requires neither intact HPV genomes nor active viral DNA replication to the high copy numbers per cell necessary for detection by less sensitive techniques such as in situ hybridization (Morris et al, 1990; Arends, 1991). Only simple preparative procedures such as xylene extraction of paraffin, proteinase K digestion, and boiling are required for analysis of these tissues, reducing the probability of loss of infrequent HPV DNA copies during processing. The high sensitivity requires meticulous precautions to minimise the risks of contamination, such as those used in this work, as well as the inclusion in every experimental run of a negative control reaction, to detect contamination should it occur, and this was exposed to the same environment during all stages of preparation, but without the addition of a template.

The detection of such tiny amounts of viral DNA raises questions as to the biological significance of its presence within lesions. It is now clear that other events are almost certainly required for HPV to contribute to malignant change within target epithelium (reviewed by Arends et al, 1990). Only a small number of copies of HPV DNA may be required for oncogenic



activity, perhaps even a minimum of one, if integrated correctly for continued expression and accompanied by other appropriate genomic changes. In support of this, it has been shown that continued expression of the E6 and E7 genes of HPV 18 within HeLa cells is associated with maintenance of the malignant phenotype, and fusion of these with normal cells suppresses both HPV 18 expression and malignancy in vivo. There is simultaneous return of both malignancy and HPV 18 early gene expression in revertants which have lost chromosome 11 (zur Hausen, 1986). HeLa cells are known to possess 30-40 copies of HPV 18, whereas another cervical carcinoma derived cell line, SiHa, contains only 1-2 copies of HPV 16 DNA, the E6 and E7 genes of which are also expressed (Pater and Pater, 1988b). This supports the hypothesis that a single HPV genome, of a high risk type, may be sufficient to contribute to the neoplastic phenotype, and an optimally specific and sensitive assay, such as this, is essential for its detection.

## 2.4 HPV DETECTION IN CONTROL TISSUE, CIN AND CARCINOMA

### 2.4.1 CLINICAL MATERIAL

Fresh control cervix was taken at autopsy from young women following accidental death, and frozen sections prepared. Frozen sections of CIN lesions were obtained from punch biopsies of abnormal aceto-white cervical lesions detected colposcopically. Samples of carcinoma were derived from paraffin blocks. Adjacent sections were taken for HPV detection, and histological assessment using sections stained with Haematoxylin and Eosin. Sections of carcinomas were also stained with Alcian Blue/Periodic Acid Schiff for mucin. The presence of normal exocervical and endocervical epithelium and glands, and the absence of abnormality, were identified in all samples of control tissue. The grade of CIN and cancer cell type were assessed independently by two pathologists, using standard criteria set out in section 2.1. The small number of cases in which there was discrepancy in CIN grade (equally distributed amongst CIN 1, CIN 2 and CIN 3), or cancer cell type, were resolved by consensus.

The PCR assay was applied to twenty cases each of CIN 1 (mean age 30.8 yr, SD 8.58, range 19-49), CIN 2 (mean age 28.65 yr, SD 7.43, range 19-45), and CIN 3 (mean age 31.4 yr, SD 8.43, range 22-50), as well as to twenty four controls (mean age 35 yr, SD 11.29, range 17-56). No significant differences in ages between any of the four

groups were found by the ANOVA or student's t tests.

A total of 47 resected cancers were analysed: 26 cases of squamous cell carcinoma (mean age 36.6 yr, SD 11.59 yr, range 20-58 yr), 17 cases of adenocarcinoma (mean 39.7 yr, SD 7.48 yr, range 28-79 yr), and 4 cases of adenosquamous carcinoma (mean 33.2 yr, SD 5.85 yr, range 26-40 yr). No significant differences in the ages of these three groups of cases and the control group were found by the ANOVA or student's t tests.

#### 2.4.2 RESULTS

No HPV DNA was detected in any of the twenty four controls. To confirm that the control autopsy frozen tissue samples and negative biopsy samples retained DNA suitable for amplification by PCR, and to exclude Taq polymerase inhibitors in these samples, they were subjected to amplification of a reference gene, the c-Ha-ras1 gene, using specific primers (Bos et al, 1987). All samples tested produced amplified DNA fragments of the appropriate size for the ras specific primers.

HPV DNA (low and high risk types) was detected in 25% of CIN 1 cases, significantly different from 60% prevalence in both CIN 2 and CIN 3 cases, and also from controls. The prevalence of individual HPV types in these lesions revealed similar frequencies of HPV 16 (45% and 55%) in both CIN 2 and CIN 3 (tables 2.2 and 2.3, figures 2.7 and 2.8).

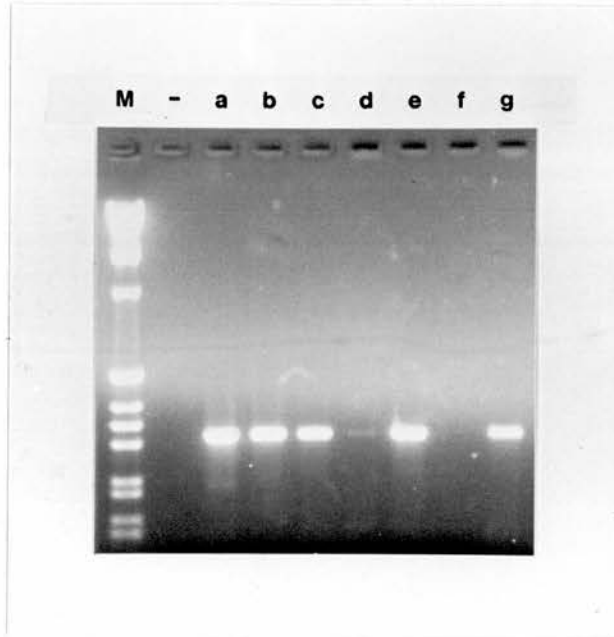


Figure 2.7

Agarose gel electrophoresis of amplified DNA from seven cases of CIN using frozen sections as template (a-g). Five are strongly positive and one (d) is weakly positive for HPV 16. These were all confirmed as type HPV 16 by oligoprobe hybridisation. One case (f) is negative. Very few "ghost bands" are generated. A "template-free" negative control track (-), and a 1kb ladder marker track (M) are included.

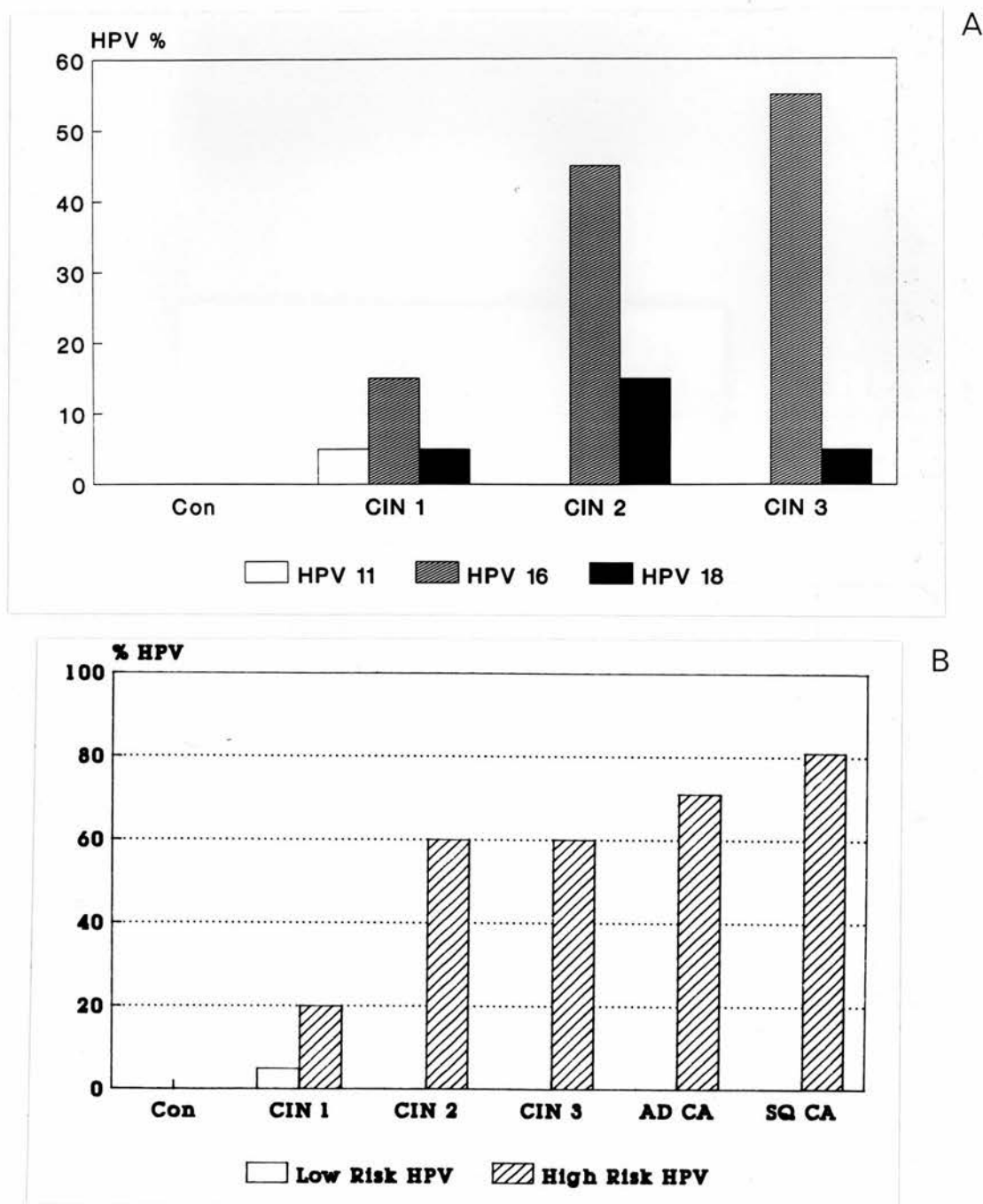


Figure 2.8

(A) Bar chart of prevalence of specific HPV types (11, 16 and 18) in twenty four controls (Con) and 20 cases each of CIN 1, CIN 2 and CIN 3. (B) Bar chart of prevalence of low risk (HPV 11) and combined high risk HPV types (16 and 18) in 24 controls (Con), 20 each of CIN 1, CIN 2, and CIN 3, 17 adenocarcinomas (AD CA) and 26 squamous carcinomas (SQ CA).

TABLE 2.2

## HPV PREVALENCE IN CONTROL TISSUE, CIN AND CARCINOMA

HPV	Con.	CIN 1	CIN 2	CIN 3	SQ.CA.	AD.CA.	A-S.CA.
Neg	24 (100%)	15 (75%)	8 (40%)	8 (40%)	5 (19%)	5 (29%)	0
6	0	0	0	0	0	0	0
11	0	1 (5%)	0	0	0	0	0
16	0	3 (15%)	9 (45%)	11 (55%)	15 (58%)	5 (29%)	3 (75%)
18	0	1 (5%)	3 (15%)	1 (5%)	6 (23%)	5 (29%)	1 (25%)
16+18	0	0	0	0	0	2 (12%)	0
33	0	0	0	0	0	0	0
Total	24	20	20	20	26	17	4

Note: Percentage proportions of totals are given in brackets.

TABLE 2.3

## STATISTICAL COMPARISONS OF HPV PREVALENCE DATA

SAMPLE 1	SAMPLE 2	$\chi^2$ Test p Value
25% CIN 1	0% Con.	<0.00001
60% CIN 2	0% Con.	<0.00001
60% CIN 3	0% Con.	<0.00001
60% CIN 2/3	25% CIN 1	0.025
81% SQ.CA.	71% AD.CA.	NS
81% SQ.CA.	0% Con.	<0.00001
71% AD.CA.	0% Con.	<0.00001
81% SQ.CA.	25% CIN 1	0.0002
71% AD.CA.	25% CIN 1	0.005
81% SQ.CA.	60% CIN 2/3	NS
71% AD.CA.	60% CIN 2/3	NS
38% HPV 16/all CIN	8% HPV 18/all CIN	0.00001
50% HPV 16/CIN 2/3	10% HPV 18/CIN 2/3	0.0001
51% HPV 16/all CA.	30% HPV 18/all CA.	<0.05
58% HPV 16/SQ.CA.	23% HPV 18/SQ.CA.	<0.05
58% HPV 16/SQ.CA.	41% HPV 16/AD.CA.	NS
23% HPV 18/SQ.CA.	41% HPV 18/AD.CA.	NS

Note: Con. = control; SQ.CA. = squamous carcinoma; AD.CA. = adenocarcinoma; A-S.CA. = adenosquamous carcinoma; Neg = Negative; 16+18 = mixed HPV 16 and HPV 18 DNA content; NS = not significant; all CA. = 26 squamous carcinomas + 17 adenocarcinomas.

High risk HPV DNA was found in 81% of squamous carcinomas (58% HPV 16, and 23% HPV 18), and in 71% of adenocarcinomas (29% HPV 16, 29% HPV 18, and 12% both types) (table 2.2, figures 2.8 - 2.10). There was no significant difference in HPV prevalence between squamous carcinomas and adenocarcinomas, whereas both differed significantly from controls and CIN 1 cases, but not CIN 2/3 (table 2.3). Closely similar prevalences were observed if analysis of adenocarcinomas was restricted to those cases without adjacent, potentially contaminating, CIN (table 2.4). In tumours of mixed differentiation, all 4 adenosquamous carcinomas contained HPV DNA (HPV 16 in 3, HPV 18 in 1).

Twenty two of the 26 squamous carcinomas were stained by the Alcian Blue/Periodic Acid Schiff method, of which 17 were mucin negative and 5 showed focal mucin positivity. These 5 consisted of 2 HPV negative, 1 HPV 16 positive, and 2 HPV 18 positive cases, indicating no obvious relationship of HPV and focal mucin positivity of squamous cancers, although only small numbers of such tumours were available. All of the negative cases were successfully amplified using primers for the reference gene c-Ha-ras1 (figure 2.11).

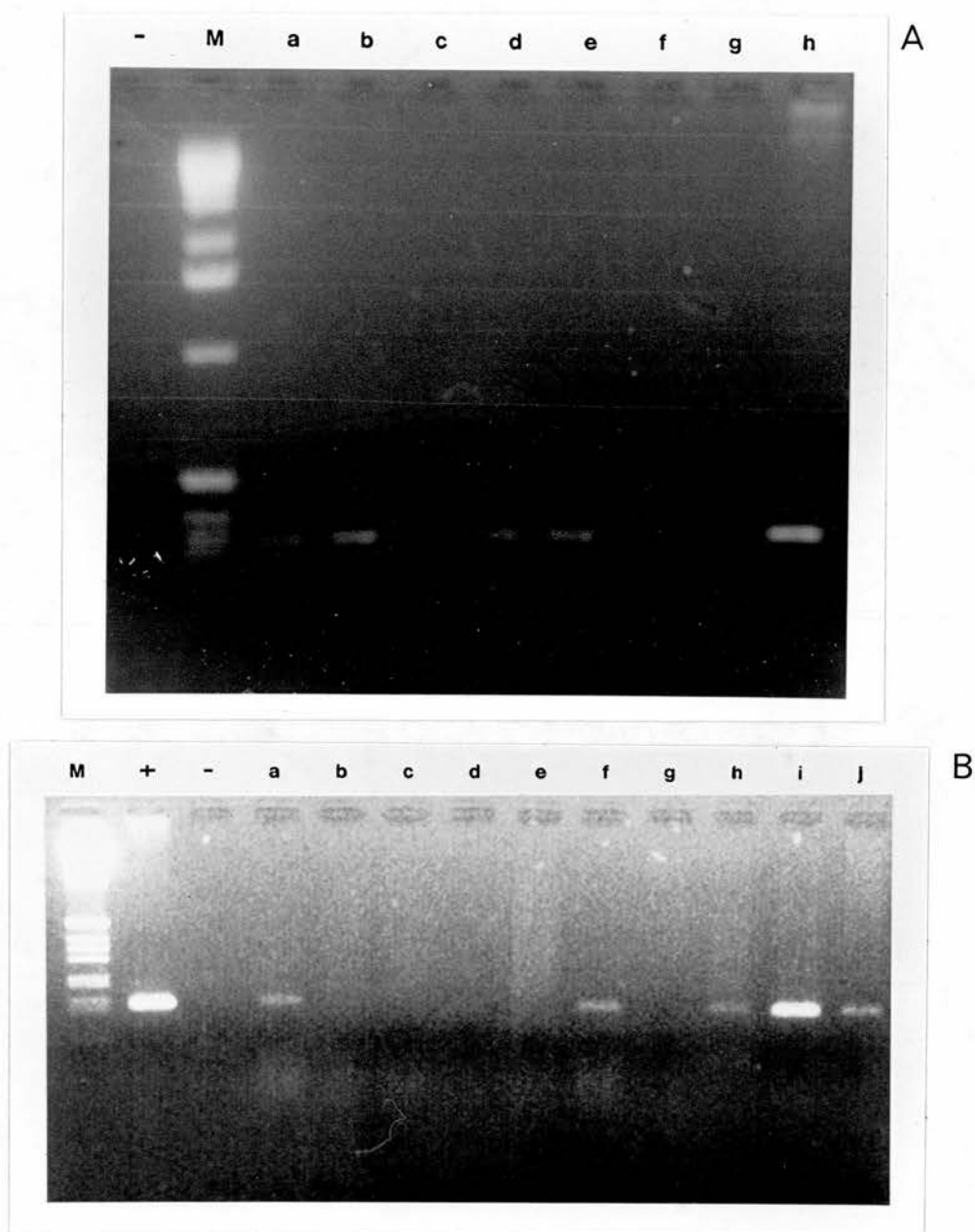


Figure 2.9

(A) Agarose gel electrophoresis of amplified DNA from PCR of 8 squamous carcinomas using primers for HPV 16. Of these cases, 5 are positive (a, b, d, e, h) and 3 are negative (c, f, g). (B) Amplified DNA from 9 adenocarcinomas (a-i) and 1 adenosquamous carcinoma (j) using primers for HPV 18. Four of the adenocarcinomas are positive (a, f, h, i), and the adenosquamous carcinoma (j) is also positive. One kb ladder marker tracks (M), plasmid DNA positive controls (+) and template free negative controls (-) are included.



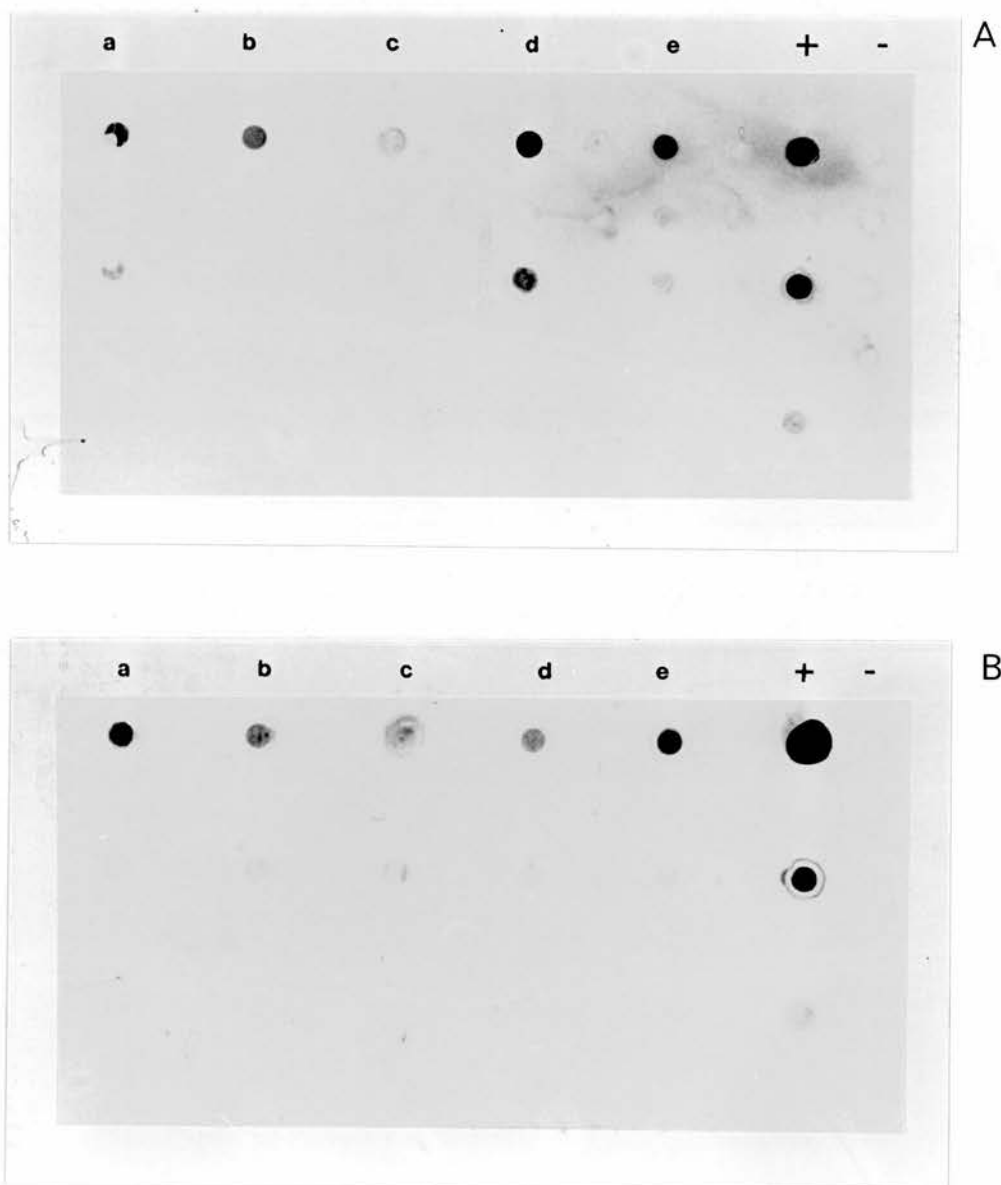


Figure 2.10

Dot blot hybridisation of PCR amplified DNA from 10 cancer cases. (A) The oligoprobe for HPV 16 shows positive hybridisation with three cases of adenocarcinoma (a, b, c), one adenosquamous carcinoma (d), and one squamous carcinoma (e). (B) The oligoprobe for HPV 18 shows positive hybridisation with four adenocarcinomas (a, b, d, e) and one adenosquamous carcinoma (c). Positive controls (+) consisting of amplified DNA from an HPV 16 plasmid, and negative controls (-) consisting of pBR322 plasmid DNA are included.

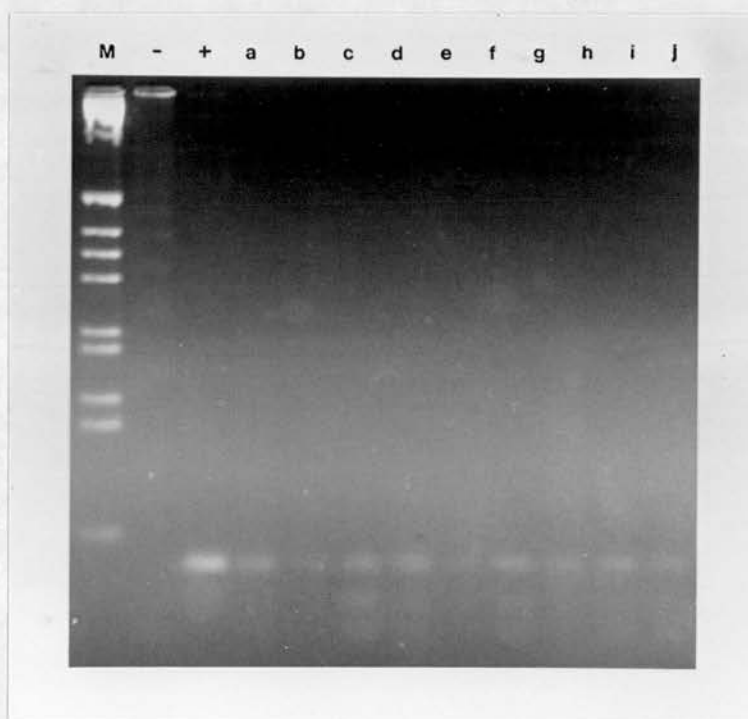


Figure 2.11

Agarose gel electrophoresis of amplified ras DNA from 10 cancer cases that were negative for HPV DNA by PCR. A set of primers for ras were used (Bos et al, 1987) that generate a 60 base pair amplified product. These were applied to four cases of adenocarcinoma (a, e, f and j), and five of squamous carcinoma (b, c, d, g and h), and all show positive ras amplification. A 1 kb marker track (M), template free negative control (-) and a ras plasmid positive control (+) are included.

**TABLE 2.4****HPV IN ADENOCARCINOMAS WITH AND WITHOUT ADJACENT CIN**

	HPV +	HPV -
ALL ADENOCA	12 (75%)	4 (25%)
AD.CA. + ADJ. CIN	4	1
AD. CA. - ADJ. CIN	8 (73%)	3 (27%)

Note: Blocks of adjacent cervical tissue were available for histological assessment of CIN in 16 out of 17 adenocarcinomas. AD.CA. = adenocarcinoma; ADJ. = adjacent. Percentage proportions are given in brackets.

In the 60 CIN cases HPV 16 was found at a significantly higher frequency of 38%, almost five-fold greater than that of HPV 18 (8%). HPV 16 also significantly exceeds type 18 in all cancers by a factor of 1.7 (51% to 30%), and in squamous cancers by a factor of 2.5 (58% to 23%). HPV 16 and 18 are equivalent in prevalence (41%) in glandular cancers, indicating a difference in relative frequency according to cancer cell type (figure 2.12, tables 2.2 - 2.6).

Possible differences in the rates of progression of CIN to cancer were compared for the two high risk HPV types, by calculating their CANCER/CIN prevalence ratios. The denominators of the ratios were HPV prevalences in premalignancies, expressed as the frequency of HPV in all CIN lesions, or just in CIN 2 and CIN 3, the immediate precursors, combined because of their identical 60% overall HPV prevalence. Grouping together CIN lesions of different grade is justified as these histological diagnoses are based on arbitrary thresholds (1/3 and 2/3 divisions of the epithelium) in a morphological continuum. The 60 CIN cases had an overall prevalence of 38% for HPV 16, significantly greater than 8% for HPV 18, whereas HPV 16 was present in 50% of the 40 CIN 2/3 cases and HPV 18 was identified in 10%, a significantly lower proportion (table 2.3). The numerators of the ratios were expressed as prevalences in all cancers (51% for HPV 16, significantly higher than 30% for HPV 18), squamous carcinomas (58% for HPV 16, significantly

greater than 23% for HPV 18), or adenocarcinomas (41% for both). The CANCER/CIN prevalence ratios were approximately two- to five-fold greater for HPV 18 than HPV 16 (table 2.5 and figure 2.13).

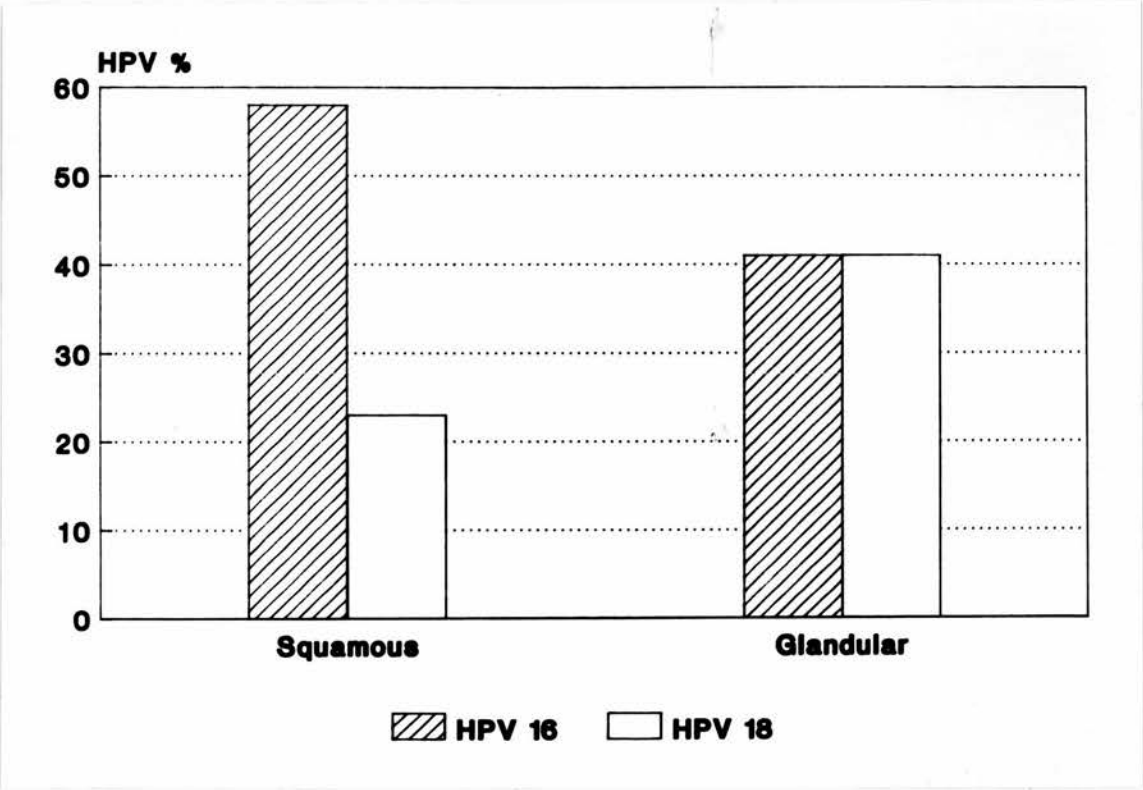


Figure 2.12

Bar chart of the prevalence of HPV 16 and HPV 18 in cancers showing either squamous or glandular differentiation.

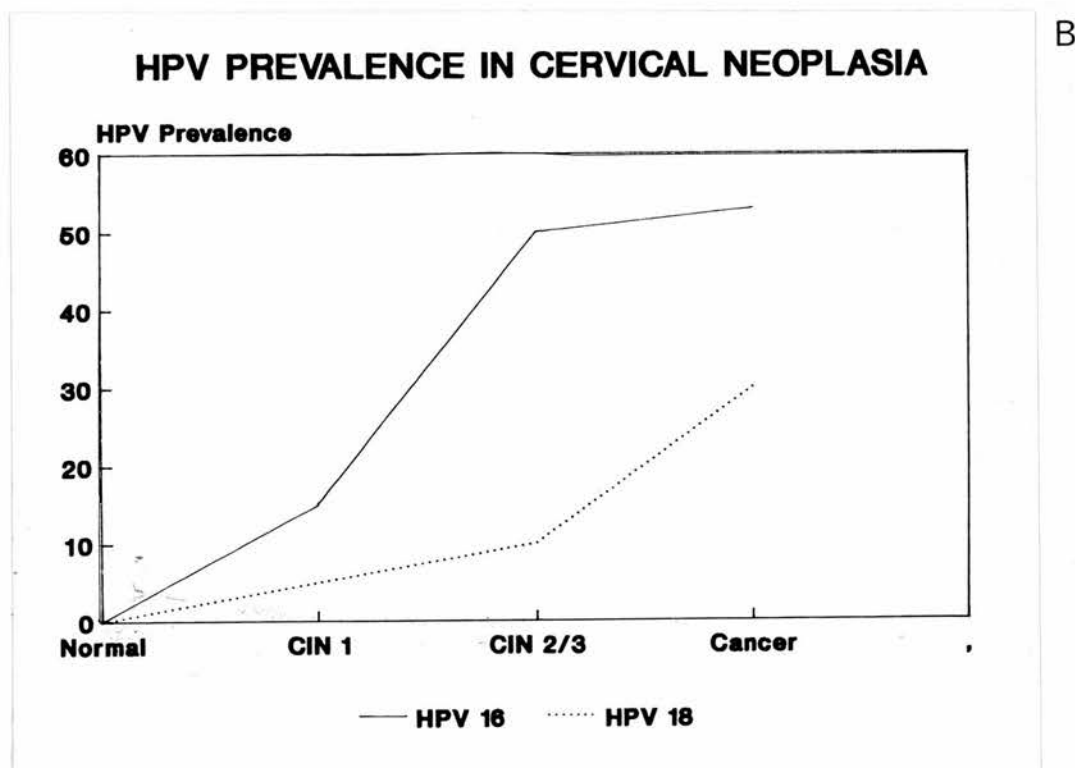
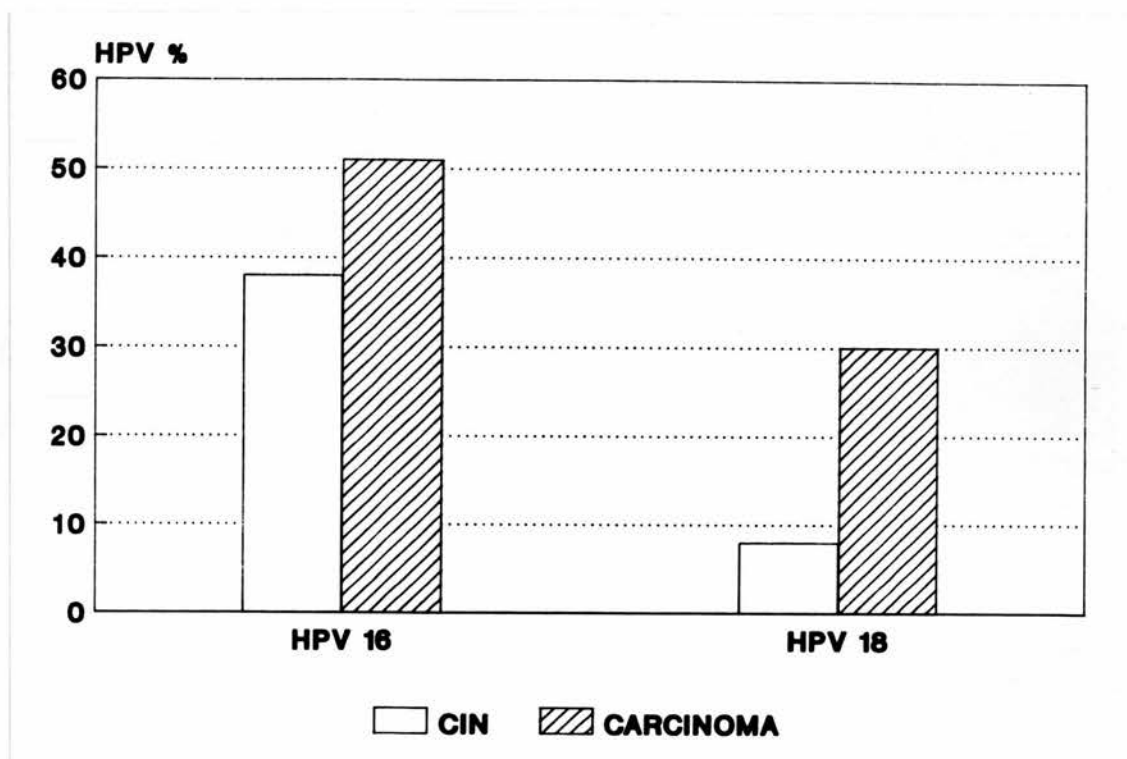


Figure 2.13

(A) Bar chart of the prevalence of HPV 16 and HPV 18 in 60 cases of CIN (20 each of CIN 1, CIN 2 and CIN 3) and in 43 squamous and glandular cancers (26 and 17 respectively). (B) Graph of the prevalence of HPV 16 and HPV 18 in 24 normal controls, 20 cases of CIN 1, 40 cases of combined CIN 2/3, and 43 cases of cancer (26 squamous and 17 glandular carcinomas). There is a marked difference in the slopes of the curves between CIN 2/3 and cancer when comparing HPV 16 and HPV 18.

**TABLE 2.5****PREVALENCE RATIOS OF CANCERS AND THEIR PRECURSORS**

PREVALENCE RATIO	HPV 16	HPV 18	18/16 RATIO
ALL CA./ALL CIN	1.3	3.8	2.9
ALL CA./CIN 2/3	1.0	3.0	3.0
SQ.CA./ALL CIN	1.5	2.9	1.9
SQ.CA./CIN 2/3	1.2	2.3	1.9
AD. CA./ALL CIN	1.1	5.1	4.6
AD. CA./CIN 2/3	0.8	4.1	5.1

Note: CA. = cancer; CIN = cervical intraepithelial neoplasia;  
 SQ.CA. = squamous carcinoma; AD. CA. = adenocarcinoma.

**TABLE 2.6****HIGH RISK HPV TYPES IN SQUAMOUS AND GLANDULAR CANCERS**

SOURCE	METHOD	HPV 16 SQ.CA.	HPV 18 SQ.CA.	HPV 16 AD.CA.	HPV 18 AD.CA.
Tase et al (1988)	ISH	54%	0%	2%	40%
Wilczynsky et al (1988)	SB	50%	7%	13%	50%
Arends	PCR	58%	23%	41%	41%

Note: SQ.CA. = squamous carcinoma; AD.CA.= adenocarcinoma;  
 ISH = in situ hybridisation; SB = Southern blotting;  
 PCR = polymerase chain reaction.



## 2.5 DISCUSSION

The data on HPV prevalence in normal cervixes, CIN and cervical cancers, obtained with this assay are qualitatively similar to many previous studies in showing high prevalence of HPV in cancer and CIN 3, but low prevalence in CIN 1 and normal cervix. However, they differ quantitatively from other reported work in several respects: a higher prevalence of high risk HPV types in CIN 2, a slightly lower prevalence in CIN 3 and cancer, different ratios of HPV 16 to HPV 18 in squamous and glandular cancers, and a complete absence of HPV in normal cervixes.

### 2.5.1 HPV IN CONTROLS

The negative results for the twenty four control samples contrast with most previous reports. The trivial explanation that autopsy material is unsuitable for PCR was excluded by amplification of a reference gene. Control prevalences, derived by PCR-based assays, have varied between 0% and 84% (table 2.7). Using non-PCR methods, 10%-12% of normal controls have been observed to be HPV DNA positive (Toon et al, 1986; deVilliers et al, 1987).

Presence of HPV DNA within cervixes of the normal population is classified as latent HPV infection, thought to occur focally within the cervix, but producing no clinical or histological effects (Schneider, 1990). These

features are likely to lead to underestimation of its prevalence if measured using small samples of localised regions of the cervix, such as the blocks tested in this study, rather than widespread scrape sampling. Thus, the method of sample collection of tissue may have tended towards underestimation.

The highest control prevalence detected by PCR was 84% (Tidy et al, 1989a and 1989b), but this was subsequently retracted, citing sample contamination as the source of error (Tidy and Farrell, 1989). Contamination may also explain some of the other high prevalences reported in the earliest PCR work, as the risks may not have been widely appreciated. Stringent precautions were taken in this study to avoid contamination, and the finding of 0% HPV positivity in the control group is in keeping with claims of their effectiveness.

Other reports of high HPV prevalence in control cervixes may reflect particular features of the patients chosen as controls. Thus, Morris et al (1989) found 53% positives in 107 cases from a high risk population taken from a sexually transmitted disease (STD) clinic, of which two thirds had abnormal cervical cytology. The assay tested for types 6, 11, 16, 18 and 33. Young et al (1989) found 70% HPV positive in only 10 cases from gynaecology or family planning clinics. These patients had at least two consecutive negative smears, but a history of cervical disease was not specifically excluded. The PCR assay

detected only types 16 and 11. The subjects chosen as controls in these 2 series may not be representative of the normal population, since all had reason to attend STD or gynaecological clinics. In contrast, the control subjects analysed in the present study were not taken from such clinics, but almost all were recruited on the apparently random basis of being victims of road traffic accidents or other causes of accidental death.

Low prevalence rates for HPV genomes present in control cervixes have also been reported. Melchers et al (1989), using a PCR method to detect the same 5 HPV types as this thesis, found 5% HPV positives in 100 cases from gynaecology clinics, with 3 consecutive negative smears and no history of cervical disease. The primers for HPV 6 and 33 were sited in L1 however, which increases the risk that integrated HPV DNA was not detected. The assay presented here amplified E6 sequences for all HPV types to avoid this risk. Van den Brule et al (1989) found 6% HPV prevalence applying a PCR assay to 220 regularly screened women in Holland. Manos et al (1990) investigated the prevalence of HPV 16 in normal populations using two PCR-based assays. One employed consensus primers situated in L1 followed by an HPV 16 type-specific probe, and the other was type-specific amplifying a segment of the HPV 16 URR (data sets I and II in table 2.7). Both assays detected identical levels of HPV 16 prevalence in three American populations: 0% in women attending planned parenthood clinics, and 11% and

22% in two geographically separate studies of female university students undergoing gynaecological examinations in health clinics.

In the control population analysed in this thesis no HPV positive samples were found using a PCR-based assay optimised for specificity and sensitivity, although only small numbers were analysed (24). This is in keeping with the 0% prevalence data from the study by Manos described above, derived from a planned parenthood clinic, and adds weight to the importance of the source of subjects.

Those controls found to be negative for HPV types 6b, 11, 16, 18 and 33 in this assay, may nevertheless contain HPVs of other types which are less well defined in terms of their oncogenic potential. Interestingly, another study of 88 control women found 11% HPV positive by Southern blotting, and 31% by PCR using consensus primers, but most of the HPV infections found only by PCR were not due to the "common genital" types (6, 11, 16, 18, 31, 33, and 45) (Schiffman et al, 1991). Manos et al (1990) also found evidence of 20% - 30% control positivity for HPV of types other than HPV 16, using an L1 consensus primer assay (data set I in table 2.7), compared with 0% - 22% for HPV 16 only (data set II), in the 3 control series described above. Thus, much of the conflicting PCR data may be explained by the method of tissue sampling, stringency of anti-contamination precautions, selection of appropriate control subjects,

and differences in design of PCR-based assays (consensus or type-specific) which influence the range of HPV types detected. There is preliminary evidence to suggest that different HPV types may be found in normal compared to neoplastic cervical epithelium. In keeping with this, the data from several groups, together with the findings presented in this thesis, are suggestive of low prevalence rates for high risk HPV types in normal cervixes. This would tend to strengthen the association of high risk HPV types with neoplastic change.

**TABLE 2.7****DETECTION OF HPV DNA IN THE CERVIX USING PCR**

REFERENCE	SPECIMEN	HPV TYPE	CIN	INVASIVE CARCINOMA	NORMAL
Shibata et al (1988)	Formalin-fixed paraffin-embedded	16, 18	95% (21)		30% (10)
Xiao et al (1988)	Formalin-fixed paraffin-embedded	16/18 16 alone 18 alone 16 & 18		90% (29) 72% (29) 48% (29) 31% (29)	
Kiyabu et al (1989)	Formalin-fixed paraffin-embedded	16, 18		74% (19)	
Melchers et al (1989)	Scrapes	6, 11, 16, 18, 33	70% (68)		5% (100)
Morris et al (1989)	Scrapes	6, 11, 16, 18, 33			53% (107)
Tidy et al (1989)	Smears	16	70% (21)	100% (20)	84% (117)
Van der Brule et al (1989)	Scrapes	6, 11, 16, 18, 33	38% (16) 60% (84)	90% (20)	6% (220) 12% (120)
Young et al (1989)	Smears	11 16	94% (38)		70% (10)
Burmer et al (1990)	Swabs	6, 11, 16, 18	67% (144)		22% (67)
Griffin et al (1990)	Formalin-fixed paraffin-embedded	6, 11, 16, 18		84% (19)	60% (10)

TABLE 2.7

## DETECTION OF HPV DNA IN THE CERVIX USING PCR ...../cont

REFERENCE	SPECIMEN	HPV TYPE	CIN	INVASIVE CARCINOMA	NORMAL
Manos et al (1990)	Swabs	I.Consensus primers include 6,11,16,18, 31,33,45 II. 16 alone			I. 31%(32) 41%(85) 44%(27) II.0%(32) 11%(85) 22%(27)
Resnick et al (1990)	Formalin-fixed paraffin-embedded	Consensus primers, include 6,11,16,18, 31,33,45 HPV 16 alone HPV 18 alone HPV 33 alone		100%(33) 76%(33) 15%(33) 3%(33)	
Shimada et al (1990)	Not stated	16,18,33 16 alone 18 alone 33 alone	70%(27)	84%(43) 77%(43) 28%(43) 14%(43)	
Ward et al (1990)	Scrapes	16	68%(19)		51%(45)
Schiffman et al (1991)	Lavage specimens	Consensus primers, include 6,11,16,18, 31,33,45	100%(12)		29%(68)

Note: PCR assays used either type-specific primers or consensus primers (key genital types detected given under HPV TYPE). Manos et al (1990) used two separate PCR methods on the same three series of normal subjects (given as data sets I and II). The numbers of cases analysed are given in brackets.



### 2.5.2 HPV IN CIN

The 60% prevalence of HPV 16/18 in both CIN 2 and CIN 3 is significantly higher than the 25% for all HPV types in CIN 1 and 0% in controls. There is also a complete absence of HPV 6b or 11 in the high grade CIN lesions. This relatively high prevalence of HPV 16 and 18 in CIN 2 (60%) is greater than reported previously for CIN 2 biopsies analysed by dot blots (Pater et al, 1986). Few PCR-based studies have included separate categories of CIN 1, CIN 2 and CIN 3, although van den Brule et al (1989) tested cervical scrapes showing mild dysplasia and found 31% HPV positive in a screened population and 58% positive in a population attending a gynaecology clinic.

The data for CIN 3 biopsies in this study are similar to the finding of 67% HPV positivity detected using PCR in a series of scrapes showing severe dysplasia (van den Brule et al, 1989). Higher prevalence rates were found in a smaller PCR study that showed 100% HPV positivity in 4 cervical scrape specimens of carcinoma in situ, and 75% HPV positivity in 8 cases of severe dysplasia (Melchers et al, 1989). Using a consensus primer PCR assay, Schiffman et al (1991) found all of 12 dysplastic scrapes contained HPV DNA, but the grades of CIN were not given.

The derivation of cells within samples influences interpretation of the data. Almost all previous PCR studies have used cervical scrapes or lavage specimens, which contain largely superficial cervical cells and



fewer basal and suprabasal cells. These methods also sample a large area of exocervical epithelium, and may include separate regions of viral replication not necessarily relevant to CIN lesions in other regions. Such widespread sampling would tend to overestimate HPV positivity in CIN. This study used tissues sampled by punch biopsy, focussed on the regions affected by aceto-white change suspicious of CIN. This confers the advantage of localising the viral agent to the abnormal cells of interest.

At the 1990 Papillomavirus Workshop, many PCR-based HPV detection studies were reviewed giving an overall prevalence of 60%-90% for all HPVs in high grade CIN lesions, with HPV 16 as the most frequent type. Undetermined HPV types (using consensus primers) were present in 10%-15% (Stanley, 1990). The report in this thesis of HPV 16 and 18 positivity in 60% of CIN 3 lesions using an absolutely type-specific assay is consistent with these data.

The findings presented here emphasise the biological similarity of CIN 2 and CIN 3 lesions in terms of the prevalence of high risk HPV types, and their divergence from CIN 1. This supports a contribution of high risk HPV types to neoplastic change in cervical epithelium, particularly the transition to lesions of higher grade than CIN 1.

### 2.5.3 HPV IN CERVICAL CARCINOMAS

The finding of HPV 16 and 18 in 81% squamous carcinomas and 71% adenocarcinomas is similar to prevalences of 70%-100% reported by others using PCR methods (table 2.7) (Xiao et al, 1988; van den Brule et al, 1989; Resnick et al, 1990; Stanley, 1990). The results demonstrate increasing prevalence of high risk HPV types through the spectrum of cervical neoplasia towards malignancy (figure 2.8).

HPV 16 was significantly more prevalent in all cervical cancers (51%) than HPV 18 (30%). If this was due to differences in overall prevalence of these HPV types in the community, and HPVs had no influence upon cancer cell differentiation, similar prevalences of HPV 16 and 18 would be expected in both squamous and glandular cancers. However, HPV 16 was found in 58% of squamous carcinomas, significantly greater than the 23% containing HPV 18, but HPV 16 and HPV 18 were present in equal proportions (41%) of glandular cancers, a much less common tumour type (figure 2.12, tables 2.2 and 2.3). Significant differences were not seen comparing the prevalences of HPV 16 in squamous versus glandular cancers, or comparing HPV 18 positivity in the two histological types of cancer. The overall pattern of HPV prevalence suggests a trend towards HPV 18 overcoming a lower overall prevalence than HPV 16 when found in adenocarcinomas. This pattern shows some similarities to previously

reported data gathered by in situ hybridisation and Southern blotting, but the more sensitive PCR assay used here detected a higher prevalence of HPV 18 in squamous cancers, and a greater HPV 16 positivity in glandular cancers (table 2.6) (Tase et al, 1988; Wilczynsky et al, 1988).

Two groups have detected little or no HPV in cervical adenocarcinomas. Young et al (1990) examined 21 cases of adenocarcinoma using in situ hybridisation and found no evidence of HPV types 16 or 18. Griffin et al (1991) tested 16 cases of adenocarcinoma using PCR and found low prevalences of HPV type 16 (25%) and type 18 (4%). In the light of the data presented here, it is much more likely that the low prevalences detected by Young and Griffin can be accounted for by methodological considerations. First, in situ hybridisation is known to be significantly less sensitive than PCR for detection of HPV (Levi et al, 1989), and Young et al gave no indication of the sensitivity of the method they used. Second, Griffin et al used a large section (50 um thick) of paraffin wax embedded tissue as the template for HPV detection by PCR assay. Others have found that when using paraffin wax embedded tissue as a template for PCR, the product yield decreased with increasing amounts of paraffin-embedded tissue (Lo et al, 1989). PCR inhibitors would be expected, given the amount of this type of tissue analysed by Griffin et al, and furthermore, negative cases were not checked for PCR

inhibitors by amplifying a reference gene such as ras DNA amplification as used in this study. Third, the possibility that samples of adenocarcinomas may be contaminated with HPV DNA from adjacent CIN was excluded in this study, as cases were assessed histologically for adjacent CIN, and no relationship between this and HPV positivity was found: 73% of cases with no adjacent CIN, and 71% of all adenocarcinomas, contained HPV DNA.

Two explanations can be offered for the differences in prevalence of HPV 16 and 18 in cervical cancers (figure 2.14). The 2 HPV types may preferentially infect different cervical cell types. Since initial infection is thought to involve basal cells of the cervical epithelium, this implies that certain cells are already committed to either glandular or squamous differentiation. There is no direct evidence to support this, and the behaviour of cervical stem cells in forming either glandular or squamous epithelium is not fully understood. Alternatively, HPV 18 infection of uncommitted stem cells in the basal layer may tend to induce glandular differentiation of subsequently formed cancers or their precursors, whereas HPV 16 infection may tend to induce squamous differentiation. Further experimental work is required to establish the validity of either proposition.

## HPV AND CANCER CELL DIFFERENTIATION

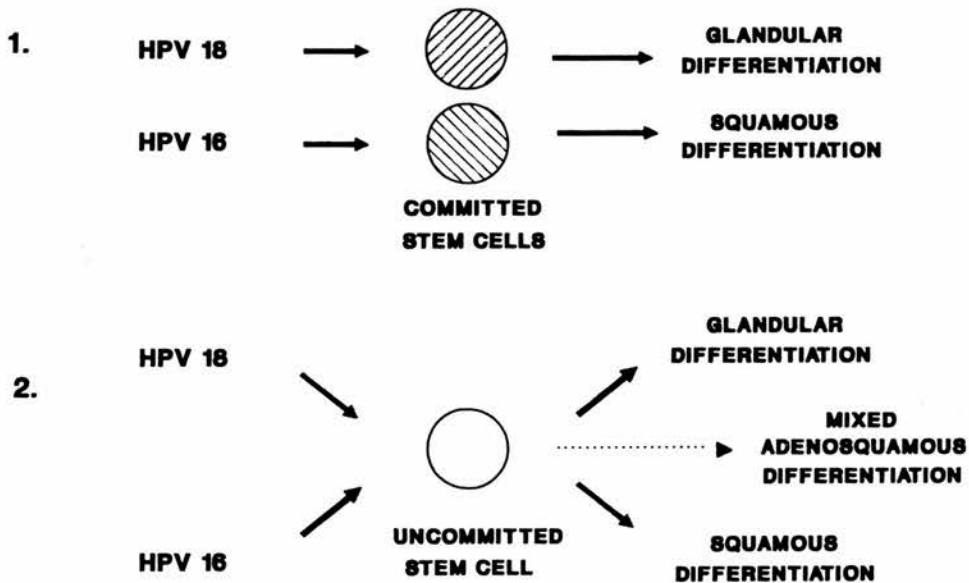


Figure 2.14

Two possible models of the relationships of high risk HPVs and cancer cell differentiation. In the first, separate HPV types preferentially infect stem cells already committed to a particular pathway of differentiation. In the second, HPVs infect an uncommitted stem cell and different HPV types influence the pattern of differentiation along alternative pathways.

Reconstructions using cervical epithelial cells transfected with HPV genomes and cultured on rafts in vitro invariably display squamous differentiation (McDougall, 1990; Meyer and Laimins, 1992). This probably reflects the influence of the raft culture conditions on the target cells used. Perhaps transfection of a different cell type may be more informative, for example embryonal stem cells which can remain undifferentiated or can differentiate according to culture conditions.

Cervical squamous carcinomas occur more frequently and have a better overall prognosis than adenocarcinomas (Buckley et al, 1988; Buckley and Fox, 1989). The association of squamous cancers with significantly more HPV 16 than HPV 18, compared with equivalence of HPV prevalence in adenocarcinomas, suggests that HPV 16 confers a better prognosis in terms of histological type of cancer, regardless of the mechanism of interaction of HPV with the programme of cell differentiation. HPV genes may influence cell differentiation, or genes controlling cell differentiation may regulate expression of HPV genes to different extents (as occurs in replicative infections and in vitro raft cultures), or a combination of the two.

HPV types may differentially affect progression of precursor lesions to cancers. It is widely accepted that squamous carcinoma can develop from CIN, and similarly adenocarcinoma in situ (AIS) may progress to

adenocarcinoma (Ferenczy and Winkler, 1987; Richart, 1987). However, much less is known about the possibility of CIN transforming to adenocarcinoma. CIN can be found adjacent to glandular cancers in a proportion of cases, 31% in this study (table 2.4), supporting the existence of this sequence of progression (Okagaki et al, 1989). In contrast, AIS is uncommon, was not identified in the material analysed here, and the possibility of its transformation to squamous carcinoma, although remote, cannot be excluded. In another study, HPV 18 was found in two thirds of 88% AIS cases positive for HPV mRNA, and one third contained HPV 16 (Farnsworth et al, 1989), supporting a role for HPV in the AIS-adenocarcinoma sequence.

CANCER/CIN prevalence ratios may shed light on different rates of progression along the precursor-malignancy sequence associated with HPV 16 and HPV 18. These may be calculated in different ways according to the type of precursor and cancer to be compared (table 2.5). The overall pattern of HPV prevalence in all cancers and all CINs gives a ratio of 1.3 for HPV 16 (51%/38%) and 3.8 for HPV 18 (30%/8%). This three-fold difference in ratio is maintained when comparing HPV prevalences in all cancers and in CIN 2/3 lesions, the more immediate precursors. This difference is demonstrated graphically by the steeper slope between CIN 2/3 and cancer for HPV 18 compared with that for HPV 16 (figure 2.13). The prevalence ratios move closer together, giving a two-fold



difference between HPV 16 and HPV 18, when comparing prevalences in squamous carcinomas either with all CINs, 1.5 for HPV 16 (58%/38%) and 2.9 for HPV 18 (23%/8%), or with CIN 2/3 lesions, 1.2 and 2.3 for HPV 16 and 18 respectively (table 2.5). These figures are broadly similar to those of Kurman et al (1988), who compared HPV prevalences in squamous cancers and those in all CINs: 1.1 for HPV 16 (41%/37%) and 7.3 for HPV 18 (22%/3%). Kurman used Southern blotting and reported lower prevalences, particularly for HPV 18 in CINs, than those described here. There are five-fold differences between HPV 16 and 18 in the prevalence ratios for adenocarcinomas and either all CINs, or CIN 2/3 lesions. This last set of ratios should be interpreted with caution as AIS may be a more important precursor of adenocarcinoma than CIN, although there is a lack of information on this question which may be related to the relative infrequency of AIS lesions. Overall, there is a consistent pattern of two- to five-fold difference between HPV 16 and HPV 18 in the range of prevalence ratios. The relatively lower frequency of HPV 18 in CIN compared with invasive cancer may represent a more rapid transit time through the precursor stage associated with HPV 18. This suggests that HPV 18 is a more aggressive type than HPV 16, in playing a role in rapidly progressive cervical neoplasia. This proposition is examined further in later sections of this thesis.

The prevalence data may provide an indication of the



position in the CIN-cancer sequence at which high risk HPVs make a key contribution and allow refinement of the model of multistage carcinogenesis set out in chapter 1 (figure 2.15). HPV types 16 and 18 have a low level of association (0%-20%) with histologically normal epithelium (latent infection) and CIN 1. A sharp increase in prevalence to 60% is observed for CIN 2 and CIN 3, and a further, smaller increment to 70%-80% for invasive cancers. The greatest increase in prevalence (3-fold) to CIN 2/3 is suggestive of the point of maximal contribution to neoplastic change. Experimental reconstructions of human genital keratinocytes transfected with HPV 16 or 18 DNA grown on raft cultures also show morphological appearances approximately equivalent to CIN 2/3 (McCance et al, 1988; Rader et al, 1990; McDougall, 1990), supporting this assignment of the point of HPV effect. This raises the question of why high risk HPVs may sometimes be associated with no histological changes, sometimes CIN 1, and at other times CIN 2/3? One possible explanation is the level of expression of the viral oncogenes E6 and E7. Low dose infection with small copy numbers of HPV genomes, or host cell suppression of viral transcription, may lead to latent infection or only minor morphological changes such as CIN 1. Greater copy numbers of HPV DNA in basal cells, or escape from host cell control of viral transcription, may produce higher levels of E6 and E7 proteins. These may reach a sufficient level to interact with cellular

oncosuppressor proteins p53 and Rb, inactivating or subverting them, to affect cell functions and result in CIN 2/3 lesions. Further deregulation of expression of E6 and E7 following viral integration, perhaps acting in cooperation with other host cell genetic changes, may produce malignant transformation to invasive carcinoma.

This model allows for both direct transition from normal to CIN 2/3, or sequential progression through latent HPV infection, or CIN 1 to CIN 2/3, according to the levels of E6 and E7 proteins. Although high risk HPVs are envisaged as playing a central role, other host cell genetic changes influencing development of CIN are not excluded, either separate from viral effects as in HPV negative CIN lesions, or in conjunction with HPVs. Growth advantage induced at the CIN 2/3 stage may increase the probability of further progression to cancer, and differences at this stage between HPV 16 and HPV 18 may explain the more rapid transition to malignancy associated with HPV 18. Such differences between the two virus types may be due to functional aspects of the E6 and E7 proteins, or perhaps differences in their relative levels of expression.

## CIN - CANCER SEQUENCE

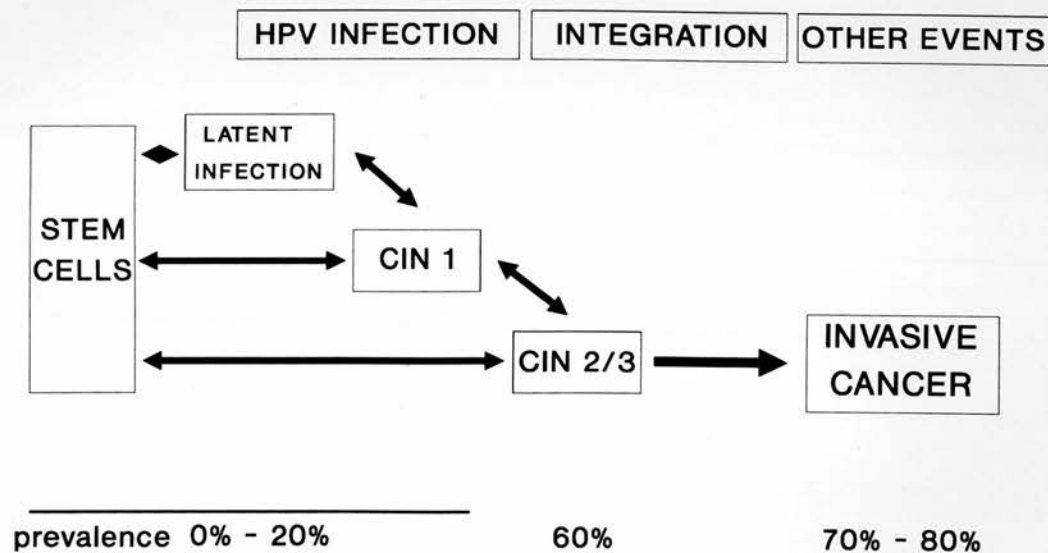


Figure 2.15

Model of events in the CIN-CANCER sequence, which incorporates the prevalence data. HPV infection of stem cells in the cervical epithelium may lead to either latent infection or CIN 1 (0%-20% prevalence of HPV 16 and 18). CIN 1 may progress to CIN 2/3, or alternatively, HPV infection may directly induce CIN 2/3 lesions (60% of which contain high risk HPV DNA). Integration of the viral genome and other events (oncogene activation and inactivation of oncosuppressor genes) are likely to be involved in the transition to invasive cancer (70%-80% of which contain high risk HPVs) and subsequent development of metastatic cancer. The precise point at which integration occurs is not certain, but this may be either before or at the time of transition to malignancy. The biggest jump in prevalence from 20% to 60% occurs at the transition from latency/CIN 1 to CIN 2/3 and this indicates the key point of action of high risk HPVs.

## 2.6 CONCLUSIONS

1) Using a PCR-based assay with many new features, the prevalence of high risk HPV types in normal cervical epithelium was found to be zero.

2) The HPV prevalence data emphasise the biological similarity of CIN 2 and CIN 3 lesions, and their divergence from CIN 1. This suggests that high risk HPVs may make a key contribution early in the neoplastic process by generating these high grade premalignant lesions.

3) HPV 16 is associated with more cancers showing squamous differentiation, and HPV 18 with the clinically more sinister glandular differentiation. Similarly, HPV 18 appears to be associated with a more rapid neoplastic progression as it has higher CANCER/CIN prevalence ratios than HPV 16. For these reasons, HPV 18 appears to be a more aggressive type than HPV 16.

### 3. GENETIC REGULATION OF TUMOUR CELL APOPTOSIS

#### 3.1 Introduction

- 3.1.1 Apoptosis in Neoplasia
- 3.1.2 Oncogenes and Tumour Cell Apoptosis
- 3.1.3 Viral Genes and Tumour Cell Apoptosis
- 3.1.4 Human Papillomavirus Transformation
- 3.1.5 Tumour Suppressor Protein Function

#### 3.2 Methods

- 3.2.1 Molecular Biology Techniques
- 3.2.2 Cell Biology Techniques

#### 3.3 Plasmid Structure and Construction

- 3.3.1 Existing Plasmids
- 3.3.2 Construction of New Plasmids

#### 3.4 Cell Line Construction and Characterisation

- 3.4.1 Transfection with Oncogene and HPV DNA
- 3.4.2 Identification of Transfected DNA
- 3.4.3 Oncogene Expression

#### 3.5 Growth Parameters of Oncogene Transfectants

- 3.5.1 Growth in Culture
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#### 3.6 Discussion

- 3.6.1 Conservation and Expression of Transfected Genes
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- 3.6.3 Apoptosis In Vitro and In Vivo
- 3.6.4 Relationship of Population Expansion in Culture with Cell Birth and Cell Death
- 3.6.5 Regulation of Apoptosis by Oncogenes and HPV
- 3.6.6 Relationship of Net Tumour Growth and Cell Turnover
- 3.6.7 Tumour Necrosis

#### 3.7 Conclusions

### 3.1 INTRODUCTION

#### 3.1.1 APOPTOSIS IN NEOPLASIA

During tumour growth, cells may be lost by a variety of means. These include cell death by apoptosis or necrosis, cell migration or exfoliation, and differentiation of tumour cells to non-proliferative terminally differentiated cells. Of the modes of cell loss, death appears to be the most numerically significant and can occur by either necrosis or apoptosis (Wyllie, 1985). Necrosis does occur at elevated levels in very aggressive tumours, but this is thought to be related to rapid expansion of tumour size producing zones of hypoxia and a lack of blood-borne nutrients (Rotin et al, 1986), variation in the production of angiogenic factors by the tumour cells (Denekamp and Hobson, 1982; Folkman, 1986) and production of tumour necrosis factors (TNF) by the host (Old, 1985; Klostergaard, 1986). Tumour necrosis has been related to the state of oxygenation of blood in the tumour environment (Schatten et al, 1962; Lala, 1972) and necrosis has been observed at a strikingly constant distance from blood vessels for any tumour (Thomlinson and Gray, 1955; Tannock, 1968; Jones and Camplejohn, 1983; Tozer et al, 1990).

High tumour cell loss, however, can occur in tumours with almost no necrosis (Lala, 1972). There is evidence that apoptosis accounts for regression not only of non-neoplastic tissues (Wyllie et al, 1973a and 1973b; Kerr

and Searle, 1973) and preneoplastic focal proliferations of hepatocytes (Bursch et al, 1984), but also of hormone-dependent carcinomas (Gullino, 1980), tumour cell death induced by anticancer agents (Barry et al, 1990; Dive and Wyllie, 1992), and experimental pancreatic and breast carcinomas after treatment with peptide hormone analogues (Szende et al, 1989). Apoptotic bodies have been observed in many human cancers, including cervical carcinoma (Searle et al, 1973), and these are rapidly phagocytosed by adjacent tumour cells and degraded by their lysosomal enzymes (Kerr and Searle, 1972a and 1972b). The paradoxically slow growth of basal cell carcinomas which have a high mitotic index, but little or no necrosis (Kerr and Searle, 1972a) is also attributable to apoptosis.

Apoptosis has been suggested to be the only cellular process capable of adequately accounting for the majority of tumour cell loss, although there is little direct quantitative evidence of this (Kerr et al, 1972; Kerr and Searle, 1972a; Wyllie et al, 1981; Wyllie, 1985; Sarraf and Bowen, 1986; Bowen and Bowen, 1990). It is not known whether tumour cell apoptosis occurs mostly in response to endogenous activation of internal genetic programmes, or whether there is a sizable contribution by exogenous stimuli, including immune cell attack, therapeutic agents, hyperthermia, toxins and relative hypoxia around necrotic zones (Wyllie, 1985; Dyson et al, 1987; Barry et al, 1990).



Little is known of the roles of oncogenes and viral genes in the regulation of tumour cell apoptosis, and these are reviewed here. Possible mechanisms of transformation by myc and ras were described in chapter 1, and those of human papillomaviruses are discussed in this section.

### 3.1.2 ONCOGENES AND TUMOUR CELL APOPTOSIS

Recently, Wyllie et al (1987 and 1989) initiated study of whether the rates of apoptosis in tumours are influenced by oncogene expression, by counting the frequency with which apoptosis is found in experimentally transplantable tumours. Oncogene expressing rodent fibroblast lines transfected with c-myc, or mutationally-activated c-Ha-ras1 (from the T24 bladder cancer cell line, with a point mutation at codon 12), or the c-Ha-ras1 proto-oncogene, formed malignant tumours in immune suppressed mice (Spandidos, 1985; Wyllie et al, 1987 and 1989). The immortalised 208F parent cell line gave rise to small, non-progressive nodules. About half of the mice injected with c-myc transfectants developed tumours at the injection site, which grew slowly, were weakly tumorigenic and non-metastatic at 14 days. Within these tumours, apoptosis was conspicuous, occurring at similarly high levels to mitosis. In contrast, over 90% of mice injected with cells expressing activated T24-ras developed aggressive, large, metastasising tumours in which apoptosis was seldom seen although mitosis occurred



at high frequency. Cells expressing the c-Ha-ras1 proto-oncogene were intermediate in properties.

The net tumour growth was consistent with the balance of cell gain and loss, manifested by the differing frequencies of mitosis and apoptosis. The T24-ras expressing cells differed from the others, both in combining low apoptosis with high mitosis, and in producing more aggressive tumours. Thus, with regard to these single examples of oncogene transfectants, there are indications that ras and myc oncogenes appear to differentially regulate the relative rates of cell gain and loss within primary tumours. Myc appears to stimulate tumour cell apoptosis, whereas ras appears to suppress it.

Others have reported similar properties of ras genes. Withdrawal of interleukin-3 induced cell death of mouse PB-3c mast cells, and this was reversed on induction of Ha-ras oncogene expression (Andrejauskas and Moroni, 1989). Ki-ras expression decreased in association with the onset of apoptosis in rat chloroleukaemic cells (Servomaa and Rytomaa, 1987). Other genes have also been implicated in the control of tumour cell apoptosis. Overexpression of the bcl-2 oncoprotein reduced apoptotic cell death of a pro-B lymphocyte cell line (Hockenberry et al, 1990), and extended the survival of certain haematopoietic cell lines after growth factor withdrawal (Vaux et al, 1988; Nunez et al, 1990).

### 3.1.3 VIRAL GENES AND TUMOUR CELL APOPTOSIS

Much is known of the effects of viral genes on tumour cell proliferation. In contrast, few studies have been performed correlating DNA viral gene activity with effects on tumour cell apoptosis. One such investigation involved the Epstein-Barr virus (EBV), a human herpesvirus. EBV is associated both with infection and transformation of B cells. Persistent infection implies a mechanism of viral persistence in B cells in vivo which usually have only a limited lifespan. EBV has also been implicated as a key aetiological agent in the African form of Burkitt's lymphoma (BL) (a B cell malignancy), and nasopharyngeal carcinoma (epithelial cancer) in China (Lenoir and Bornkamm, 1987; Rickinson, 1990). EBV latent proteins have been shown to suppress apoptosis in Burkitt's lymphoma clones (Gregory et al, 1991). Thus, inhibition of tumour cell apoptosis, by certain viral genes, may be an important contributor to oncogenic growth.

The possibility also exists that HPV influences apoptosis of its target cell, either during infection altering the lifespan of the infected cell, or following transformation affecting the rate of population expansion of neoplastic cells. This hypothesis is explored in this thesis. The molecular mechanisms of HPV-induced cell transformation may also be relevant to viral control of tumour cell gain and loss, and these will be reviewed.

#### 3.1.4 HUMAN PAPILLOMAVIRUS TRANSFORMATION

Studies of transformation by bovine papillomavirus (BPV1) and other animal papillomaviruses (Howley et al, 1986), established the carcinogenic properties of this group of DNA viruses and set out the systems for analysis of transforming potential of human PVs once these became available as cloned genomes. Early experiments demonstrated that human papillomaviruses 5, 16 and 18 were capable of transforming to tumorigenicity, the established rodent cells C127 or NIH3T3 (Watts et al, 1984; Yasumoto et al, 1986; Bedell et al, 1987). Subsequently, high risk genital HPV types 16, 18, 31 and 33, but not low risk HPV types 6b and 11, were shown to cooperate with mutationally activated human c-Ha-ras1 to transform primary rodent kidney epithelial cells to malignancy (Pater et al, 1988a; Storey et al, 1988; Crook et al, 1988). Primary rodent cell transformation in cooperation with high risk HPV types is also observed with v-fos (from the FBJ murine sarcoma provirus), but not v-fms, v-mos, c-src or the human p53 minigene (Crook et al, 1988).

The natural target cell of HPV in vivo is, of course, not the rodent fibroblast or kidney epithelial cell, but the keratinocyte. HPV alone does not transform primary human keratinocytes or other cell types to tumorigenicity. However, experimental introduction of DNA of HPV types 16, 18, 31 or 33, but not types 6b or 11,

into human foreskin or cervical keratinocytes induces several key attributes of the neoplastic phenotype. These include immortalization (rescue from senescence), aneuploidy and a reduced growth factor requirement (growth in low serum) (Durst et al, 1987b; Pirisi et al, 1987 and 1988; Woodworth et al, 1988 and 1989). HPV 18 DNA is 5 times more efficient than HPV 16 DNA at immortalizing human keratinocytes, regardless of the method of transfection, indicating the more aggressive properties of HPV 18 (Barbosa and Schlegel, 1989).

HPV 16 or 18 transfected keratinocytes retain the ability to stratify, but fail to differentiate normally when cultured on "dermal equivalent" collagen rafts in vitro, and show histological abnormalities similar to genital intraepithelial neoplasia in vivo (McCance et al, 1988; Rader et al, 1990). These stratified raft cultures show downregulation of differentiation-specific keratins (such as K6a and K14), and increased resistance to differentiation signals, such as calcium and TPA (Kaur and McDougall, 1989). Complete loss of differentiation was observed in late passage HPV 18 immortalized cells (Hudson et al, 1990), and one HPV 18 immortalized line has been derived which is tumorigenic at high (>60) but not low (<20) passage number (McDougall, 1990).

The addition of mutationally activated v-Ha-ras or v-Ki-ras to HPV 16-immortalized human keratinocytes results in full malignant transformation, with production of

squamous cell carcinomas on injection into immunologically incompetent mice (DiPaolo et al, 1989; Durst et al, 1989).

The E6-E7 subgenomic fragment has been shown to encode the transforming functions of HPV 16 and 18 in established rodent cell lines (Matlashewski et al, 1987; Bedell et al, 1987; Storey et al, 1988; Vousden et al, 1988 and 1989), and also the immortalizing functions in primary human genital keratinocytes (Kaur et al, 1989; Munger et al, 1989a). Studies on transforming activities of single genes, subcloned from high risk HPV types, showed that the activity of E6 was lower than that of E7 (Kanda et al, 1988; Vousden and Jat, 1989; Storey et al, 1988; Crook et al, 1988). E7 genes from low risk HPV types 6b and 11 show markedly reduced transforming activity by comparison (Storey et al, 1990). E7 but not E6 can immortalize human genital keratinocytes - although the efficiency is enhanced by E6 (Hudson et al, 1990). The E7 and E6 genes apparently influence different aspects of the transformation phenotype in other experimental models (Yutsudo et al, 1988). Both E6 and E7 sequences were required for HPV 16 induced extension of life span of human fibroblasts (Watanabe et al, 1989). The E6-E7 region may not be the sole transforming domain, as transfection experiments with rodent NIH3T3 cells have demonstrated a lower level of transforming activity in the E2-E4-E5 region of both HPV 16 and 18 (Vousden et al, 1988; Bedell et al, 1989). This

activity may reside in the E5 gene, which is known to have a transforming function in BPV1, and has been shown to activate, by tyrosine phosphorylation, the membrane receptors for epidermal and platelet-derived growth factors (Martin et al, 1985; Petti et al, 1991). BPV 1 E5 also binds to the 16Kd component of vacuolar H<sup>+</sup>-ATPases critical for the function of cellular compartment processing of membrane-associated molecules such as growth factor receptors (Goldstein et al, 1991).

Differences in oncogenic potential of high and low risk HPV types have been investigated by comparison of the nucleotide sequences of HPV 16 and 18 with those of HPV 6b and 11. This has revealed that within the E6 sequences of the two high risk viruses there are potential internal RNA splice sites, both donor and acceptor. When used by the host cell RNA splicing machinery in the processing of E6 containing transcripts, smaller E6 proteins (E6\*, E6\*\* and E6\*\*\*) are produced with different carboxy termini (Schneider-Gadicke and Schwar z, 1986; Schneider-Gadicke et al, 1988; Cornelissen et al, 1990). E6 splicing may influence the rate of translation initiation from the E7 start codon (Sedman, personal communication). HPV 6b and 11 can also generate E7 mRNA by a different mechanism from HPV 16 and 18, by using a promotor within the E6 sequence, which is not present in HPV 16 and 18 E6 sequence (Smotkin et al, 1989). It remains to be evaluated whether these sequence differences in the E6 genes contribute a higher



oncogenic potential upon HPV 16 and 18, confer some other function upon them, or are merely harmless relics of viral evolution.

Experimental reconstructions therefore, strongly suggest that E6 and E7 are the viral transforming genes. Three strands of further evidence support the view that E6 and E7 sequences are the HPV genes which confer some of the oncogenic properties upon human tumour cells.

**(1) E6 and E7 sequences are conserved on integration.**

There is a consistent pattern of viral DNA integration in cervical carcinomas and cell lines, which conserves intact URR-E6-E7 DNA sequences and allows continued expression of the E6 and E7 genes (see chapter 1).

**(2) E6 and E7 proteins are expressed in neoplastic cells.**

Both human cervical carcinomas and carcinoma derived cell lines express mRNA and proteins of E6 (11-18 kDa protein) and E7 (12-15 kDa protein) (Smotkin and Wettstein, 1987; Baker et al, 1987; Seedorf et al, 1987; Androphy et al, 1987; Shirasawa et al, 1987). Human cervical cells, both immortalized by high risk HPV DNA (16, 18, 31 or 33), and malignantly transformed in 2 stages by HPV 16 and activated ras, express E6 and E7 proteins (Woodworth et al, 1989; DiPaolo et al, 1989).

**(3) E6 and E7 bind DNA and interact with tumour suppressor proteins.** Both HPV E6 and E7 amino acid sequences contain a conserved pattern of repeating cysteine doublets (Cys-X-X-Cys), similar to those found in steroid receptor zinc finger proteins, which mediate coordination of zinc, and DNA binding (Baker, 1987; Cole and Danos, 1987; Barbosa et al, 1989; Grossman et al, 1989; Grossman and Laimins, 1989; Vousden et al, 1989). Cysteine mutations in the HPV 16 E7 Cys-X-X-Cys motifs also decrease E7 transformation and transactivation efficiency (Storey et al, 1990).

The HPV 16 E7 amino acid sequence contains a motif shared with the SV40 large T antigen, the conserved domains 1 and 2 of adenovirus E1a protein, and the v- and c-myc oncoproteins. This motif has been shown to encode the transforming function of SV40 large T antigen and the specific binding of the 105 kDa protein encoded by the retinoblastoma tumour suppressor gene (p105Rb). In addition, the homologous regions of SV40 large T, E1a and c-myc proteins are required for cotransformation of primary cells with ras oncogenes (Phelps et al, 1988; Figge and Smith, 1988; Figge et al, 1988; Dyson et al, 1989). This motif, which has been referred to as the cell division or CD motif, is present in the E7 protein sequences of genital HPVs associated with malignant tumours (types 16, 18, 31 and 33), but not those associated with benign tumours (types 6b and 11), in which the first amino acid at the N terminus of the CD



motif does not match (Figge and Smith, 1988; Arends et al, 1990b). HPV 16 and 18 E7 proteins bind with high affinity to p105Rb (HPV 18 has a greater affinity than HPV 16), whereas HPV 6b and 11 E7 proteins bind with significantly lower affinities (Phelps et al, 1988). The HPV 16 amino acid sites critical for binding map to the N terminus of the CD motif (Munger et al, 1989b and 1991; Barbosa et al, 1990).

The C terminus of the CD motif serves as a substrate for casein kinase (CK) II, which phosphorylates 2 serine residues at this site, replacement of which by non-phosphorylatable amino acids reduces but does not abolish transforming activity, and does not affect p105Rb binding (Barbosa et al, 1990). Phosphorylation rates are faster for HPV 18 E7 than for HPV 16 E7, which in turn is faster than that for HPV 6b E7. Rb binding and phosphorylation are independent activities which are required for efficient transformation. E7 can also transactivate the adenovirus E2 promotor, and this function maps to an overlapping domain (Chesters et al, 1990; Watanabe et al, 1990; Storey et al, 1990). Neither E7 phosphorylation nor E7 transactivation are required for E7 transformation, but transformation is associated with Rb binding (Edmonds and Vousden, 1989; Storey et al, 1990).

The ability to bind the Rb oncosuppressor protein has been demonstrated for two other DNA tumour virus oncoproteins: SV40 large T antigen (SVLT) and adenovirus

E1a protein (AdE1a) (Whyte et al, 1988; DeCaprio et al, 1988). HPV E7, SVLT, and AdE1a all share the ability to immortalize cells, induce cellular DNA synthesis and transcriptionally transactivate various viral or cellular promoters. Mutational analysis of these proteins has shown that the CD motif acts as a specific region within E7, SVLT, and E1a, which is necessary for Rb binding (Dyson et al, 1989). Mutant forms of E7 protein which are unable to bind Rb also cannot transform or immortalize cells in culture (Barbosa et al, 1990).

Viral transforming proteins synthesised by the same three viruses also bind a second cellular tumour suppressor protein, namely p53. p53 was first identified by its binding to the SV40 large T antigen (Lane and Crawford, 1979). The adenovirus protein E1b can complex with p53 (Sarnow et al, 1982), and an in vitro association between HPV E6 and p53 has been described (Werness et al, 1990). This association is type-specific, in that HPV 16 and 18 E6 proteins bind the p53 tumour suppressor protein, whereas the E6 proteins of HPV 6b and 11 do not (Werness et al, 1990). This interaction produces increased degradation of p53 by a ubiquitin-dependent protease system (Scheffner et al, 1991).

### 3.1.5 TUMOUR SUPPRESSOR PROTEIN FUNCTIONS

Possible roles for the two tumour suppressor genes Rb and p53 include regulation of the cell cycle, perhaps

negatively controlling entry or progress through it. The p105Rb product is a nuclear phosphoprotein with DNA binding activity (Lee et al, 1987). The phosphorylation state of Rb changes with the phases of the cell cycle, and the master cell cycle kinase p34<sup>cdc2</sup> can phosphorylate Rb (Lewin, 1990). The Rb protein is not phosphorylated in G0 or G1 phase cells but becomes phosphorylated as the cells enter S phase (Buchkovich et al, 1989). It has been suggested that the unphosphorylated form of Rb is the active form, in preventing progression through the cell cycle, and this is supported by the observation that SV40 large T antigen only binds the unphosphorylated form of Rb (Ludlow et al, 1989). Senescent and quiescent fibroblasts are known to contain unphosphorylated Rb, which is phosphorylated in quiescent cells after serum stimulation, but is apparently irreversibly dephosphorylated in senescent cells (Stein et al, 1990).

The growth inhibitory effects of TGF beta on epithelial cells may be the result of suppression of Rb phosphorylation (Laiho et al, 1990), and the down-regulation of c-myc expression in keratinocytes by TGF beta is also mediated through Rb (Pietenpol et al, 1990). Rb binds to at least seven nuclear proteins, including the cellular transcription factor E2F (or DTRF) and c-myc, through a common "pocket" that mediates binding to the viral oncoproteins SVLT, AdE1A, and HPV 16 or 18 E7 (Wagner and Green, 1991). Rb has also been implicated in

negatively regulating expression of c-fos, another nuclear protein important in the control of tumour cell growth (Robbins et al, 1990).

Rb was originally identified as the genetic locus undergoing homozygous deletion in heritable retinoblastomas (Knudson, 1971), and it is now regarded as a prototype tumour suppressor gene. This is clearly demonstrated by experiments in which wild-type Rb gene sequences have been transfected into tumorigenic retinoblastoma cells lacking functional Rb, and producing reversion of the tumorigenic phenotype (Huang et al, 1988).

p53 appears to have both positive and negative activities in regulating cell cycle activity. Wild-type p53 can suppress cellular transformation by a variety of oncogenes (Finlay et al, 1989), and transfection of wild-type p53 into colorectal carcinoma cells also blocks their cell division (Baker et al, 1990). Wild type p53 has been implicated in promoting cell differentiation (Stanbridge, 1990), mediating growth arrest by growth inhibitory cytokines (Deiss and Kimchi, 1991), and inducing apoptosis in leukaemic cells (Yonish-Rouach et al, 1991). Many tumour cells appear to have lost wild-type p53 function, in keeping with the role of wild-type p53 in negative growth control, but many other tumour cell types express mutant p53 proteins which can exert dominant transforming activity.

It has been suggested that p53 can adopt at least two differing conformational states which may be identified using different antibodies (Gannon et al, 1990). One conformation appears to be associated with wild-type p53 protein, the other conformation with dominantly transforming mutant p53 protein, and also transiently by the wild-type protein during its function as a positive regulator of cell growth (Milner et al, 1990). It may be that the dominantly transforming p53 mutants have been frozen in the second conformation resulting in the loss of the negative control function and continuous expression of the positive control function. The inactivation of wild-type p53 by E6-induced degradation might have the same consequences as the deletion of the p53 locus frequently observed in many tumours. It also explains why the E6/p53 complex, which was originally described and studied in vitro, has been so difficult to demonstrate in vivo. The importance of both the E6-p53 and E7-Rb interactions is further suggested by the correlation between ability to bind these cellular tumour suppressor proteins and the oncogenic potential of the HPV type: high affinity binding is associated with high risk HPV types (Munger et al, 1989b; Werness et al, 1990).

In summary, high risk HPV types can immortalize human genital keratinocytes, and induce similar morphological changes to intraepithelial neoplasia when these cells are

grown as raft cultures. E6 and E7 are the viral transforming genes, and their products interact directly with cellular oncosuppressor proteins p53 and Rb. Mutationally activated ras can transform such cells to malignancy. It is not known how these genes influence tumour cell growth, in particular the balance of cell gain and cell loss. One approach to these problems would be to study cervical cancers from patients. However, authentic human tumours are often heterogeneous in genotype and phenotype, and this may complicate analysis of the growth kinetics. In this thesis, monoclonal cell lines were constructed to provide homogeneous populations of cells, eliminating this possible source of variability, and allowing correlation of changes in growth parameters with specific genetic manipulations. The genes investigated were myc, ras and HPV sequences. Rodent fibroblasts were selected for ease of culture, genetic manipulation and study of cell turnover both in vitro and in vivo.

## 3.2 METHODS

### 3.2.1 MOLECULAR BIOLOGY TECHNIQUES

#### 3.2.1.1 PLASMID DIGESTION BY RESTRICTION ENDONUCLEASES

Plasmid DNA was digested by the relevant restriction enzyme and the appropriate ionic strength buffer in sterile Eppendorf tubes. 10 x stock buffer solutions were used and diluted 1:10 with DDW in the final reaction. Sufficient enzyme was used to ensure complete digestion, usually 10 units of enzyme per  $\mu$ g of plasmid DNA for 2 hours at the appropriate temperature. The amount of enzyme was kept below 10% of the final volume however, as glycerol present in the enzyme storage buffer may interfere with enzymatic activity.

#### 3.2.1.2 GEL ELECTROPHORESIS

Gel electrophoresis was performed to confirm that DNA digestion had occurred, to further characterise plasmids or to isolate specific DNA fragments. Loading buffer (0.25% Bromophenol Blue, 1% Ficoll, 50% Glycerol, 0.1% SDS, 25mM EDTA at pH 8.0) was added to DNA samples (diluted 6-fold), before these were loaded into sample wells of 0.7% or 1.0% agarose gels, composed of electrophoresis grade or low melting temperature agarose, in x1 TBE buffer. One  $\mu$ l of 1 Kilobase Ladder Marker (Gibco BRL) was usually included on the gel as a size marker. Following electrophoresis at the appropriate



potential difference/current and time (4-18 hours) to separate DNA fragments of various sizes, ethidium bromide at 0.5ug/ml was used to stain the DNA with visualisation under UV light.

#### 3.2.1.3 ISOLATION OF DNA FRAGMENTS

DNA fragments within low melting temperature agarose gels were visualized under UV light, the correct band identified, excised and transferred to an Eppendorf tube. Three volumes of TE (10mM Tris (pH 8.0) and 1mM EDTA) were added and the tube incubated at 65°C for 15 minutes to melt the agarose. To this was added an equal volume of phenol, pre-warmed to 65°C, equilibrated with TE and supplemented with 0.1% hydroxyquinolone and 0.2% beta-mercaptoethanol. The sample was vortexed vigorously and centrifuged at 13,000rpm for 5 minutes. The upper aqueous phase was transferred to a fresh tube and to this was added a half volume of phenol (equilibrated as above) and a half volume of 24:1 (v/v) mixture of chloroform and isoamyl alcohol. The sample was again vortexed, centrifuged at 13,000rpm for 5 minutes and the upper aqueous phase transferred to a new tube. An equal volume of chloroform: isoamyl alcohol was added, the tube vortexed, centrifuged at 13,000rpm for 5 minutes and the upper aqueous phase transferred once more. The DNA was precipitated from the resulting aqueous solution with a half volume of 7.5M ammonium acetate and 2 volumes of absolute ethanol. This was mixed and maintained at -20°C



for 60 minutes before being centrifuged at 13,000rpm for 20 minutes at 4°C. The resulting pellet was dried in a vacuum dessicator and resuspended in TE.

#### 3.2.1.4 LIGATION OF DNA FRAGMENTS

Plasmids containing the insert and vector DNA fragments to be ligated were digested with appropriate restriction enzymes to generate the desired fragments of DNA with overhanging cohesive termini. These fragments were isolated from low melting temperature agarose gels, and small samples were electrophoresed on an agarose gel with a known amount of Hind III digested lambda DNA to provide a measure of their concentration, in order to produce a **J:I** ratio of >1 and <3 for the ligation reaction. The ratio of concatemeric ligation products depends on the parameters **J & I**, where **J** is a measure of the length of the DNA molecule and **I** is a measure of all complementary termini in the solution. An equal or slightly greater concentration of insert to vector DNA is also required to favour intermolecular as opposed to intramolecular ligation.

0.2ug of vector DNA in 1-3ul was placed in a sterile microfuge tube, 0.2ug of insert DNA in 1-3ul was added, and DDW to 8ul. The solution was warmed to 45°C for 5 minutes to melt any cohesive termini that had re-annealed and then chilled to 0°C. 10 units T4 DNA ligase enzyme in 1ul, 1ul x10 T4 DNA ligase buffer (final x1

composition: 20mM Tris-HCl at pH 7.6, 10mM MgCl, 10mM dithioerythritol, 6mM ATP) were added and the reaction incubated at 14°C for 18 hours. Two control reactions were set up with the vector DNA alone, and the insert DNA alone. 10ul of the ligation reactions were used to transform competent bacteria.

#### 3.2.1.5 PREPARATION OF COMPETENT BACTERIAL CELLS

E.coli (strain HB101) competent cells for introduction of exogenous DNA were prepared by collection during the mid-logarithmic phase of growth. A colony of E.coli bacteria freshly grown on a nutrient agar plate was used to inoculate 10ml of LB medium (Luria-Bertani medium: 10g Bacto-Tryptone, 5g Bacto-yeast, 10g Sodium Chloride, per litre of DDW, autoclaved before use), and incubated at 37°C for 18 hours with rotational mixing at 225rpm. 500ml of LB medium was inoculated with this 10ml bacterial culture and similarly incubated for 2-3 hours until the cell density approached  $1 \times 10^8$  cells/ml (optical density at 550nm of 0.2-0.4). The cells were centrifuged at 4,000g for 5 minutes and the resulting pellet was resuspended in freshly prepared TSB (Transformation Buffer: 10% Polyethylene Glycol 3000, 5% DMSO, 10mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>). The cells were placed on ice for 1 hour and dispensed into sterile, pre-cooled Eppendorf tubes in 200ul aliquots.

Known amounts of DNA were used to ascertain the

transformation of the competent cells. 10ng, 1ng, and 0.1ng of pUC19 were used as controls, the number of resultant colonies counted and the efficiency was calculated. Successful transformations occurred with formation of  $10 \times 10^5$  or more control colonies per ug of DNA.

10ul of the ligation reaction containing DNA was added to an aliquot of 200ul of competent bacterial cells. This suspension was held on ice for 30 minutes, heat-shocked at 42°C for 2 minutes, and chilled on ice for 2 minutes. 0.9ml of TSB supplemented with 20ul of 1M glucose was added to the suspension and this was incubated at 37°C with rotational mixing at 225rpm for 1 hour. The cells were concentrated by a 30 second pulse of centrifugation at 13000rpm and the supernatant was decanted. The cells were resuspended using a pipette in a volume of 200ul TSB, which was plated out onto a LB agar plate with appropriate antibiotics (50ug/ml ampicillin and/or 400ug/ml hygromycin B) using a glass rod. This was incubated at 37°C for 20 hours and antibiotic resistant colonies were isolated and inoculated onto a master LB agar plate with antibiotics (50ug/ml ampicillin and/or 400ug/ml hygromycin B).

#### 3.2.1.6 SMALL SCALE PREPARATION OF PLASMID DNA

A single bacterial colony (from the master plate) was transferred into 1ml of LB medium containing appropriate

antibiotics (50ug/ml of ampicillin and/or 400ug/ml hygromycin B) and incubated for 18 hours at 37°C with rotational mixing at 225rpm. This was centrifuged at 13,000rpm for 30 seconds at 4°C and the supernatant aspirated. The bacterial pellet was resuspended, by vigorous vortexing, in 100ul of ice-cold solution MI (50mM glucose, 25mM Tris.Cl at pH 8.0, 10mM EDTA), containing 5mg/ml of lysozyme (Sigma), to digest bacterial cell wall. 200ul of freshly prepared solution MII (0.2M NaOH, 1% SDS) was added, and the suspension mixed by inversion several times before cooling on ice. 150ul of solution MIII (3M Potassium acetate/2M Acetic acid (11.5ml Glacial acetic acid in 100ml solution) was added, to precipitate chromosomal DNA and bacterial protein, and this solution was vortexed and stored on ice for 5 minutes. The solution was then centrifuged at 13,000rpm for 5 minutes at 4°C, and the supernatant, containing plasmid DNA, was transferred to a fresh tube.

An equal volume of phenol:chloroform isoamyl alcohol was added, (25;24:1) the solution was vortexed and centrifuged at 13,000rpm for 2 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and the DNA was precipitated with 2 volumes of absolute ethanol at room temperature and centrifuged at 13,000rpm for 5 minutes at 4°C. The supernatant was gently aspirated and the pellet dried before being redissolved in 50ul of TE (pH 8.0) containing DNAase-free pancreatic RNAase at 20ug/ml and stored at -20°C. This DNA was analysed by

restriction enzyme digestion and gel electrophoresis. Clones showing the correct restriction maps were identified and their bacteria (from the master plate) were grown up on a large scale as outlined below, for use in further experiments.

#### 3.2.1.7 LARGE SCALE PREPARATION OF PLASMID DNA

This protocol is a larger scale version of the method previously described. A single bacterial colony was inoculated into 10ml of LB medium containing appropriate antibiotics (50ug/ml of ampicillin and/or 400ug/ml hygromycin B) and incubated for 18 hours at 37°C with rotational mixing at 225rpm. This was transferred to 500ml of LB medium containing appropriate antibiotics and incubated for 18 hours at 37°C with rotational mixing at 225rpm. After centrifugation at 5,000rpm for 10 minutes at 4°C, the bacterial pellet was resuspended in ice-cold 100ml NTE (100mM NaCl, 10mM Tris (pH 8.0), 1 mM EDTA solution), and immediately centrifuged at 5,000rpm at 4°C for 10 minutes. The bacterial pellet was resuspended in 10ml of solution MI containing 5mg/ml of lysozyme (Sigma), and held on ice for 5 minutes. 20 ml of solution MII was added and the suspension was mixed by gently swirling several times before standing on ice for 5 minutes. 15ml of ice-cold solution MIII was added, the mixture was inverted three times, and maintained for 30 minutes on ice. It was centrifuged at 8,000rpm for 20 minutes at 4°C, the high molecular weight DNA and

bacterial debris forming a pellet. The supernatant was transferred to a new tube and the DNA precipitated by adding 1 volume of ice-cold isopropanol, and maintaining on ice for 30 minutes, before centrifugation at 12,000g for 30 minutes at 4°C. The resulting DNA pellet was washed with 70% ethanol, dried in a vacuum dessicator, and resuspended in a total volume of 4ml of TE (pH 8.0).

A caesium chloride-ethidium bromide gradient was prepared by the addition of 4.0g of caesium chloride and approximately 400ul of 10mg/ml ethidium bromide while adjusting the refractive index to 1.392. This was transferred to a Beckman polyallomer tube, sealed, and spun in an ultracentrifuge at 38,000rpm for 60 hours at 20°C. This produced separation of plasmid DNA into two bands, the upper band of nicked circular and linear plasmid DNA (also containing any residual bacterial DNA) and the lower band of closed, circular plasmid DNA. The lower band was extracted and transferred to a fresh tube. An equal volume of butanol (saturated with TE) was added and after centrifugation at 13,000rpm for 5 minutes, the upper phase of butanol containing ethidium bromide was drawn off and a fresh volume added. This was repeated until no ethidium bromide dye was observed in either layer (approximately three to five times). The aqueous phase containing DNA was removed and the DNA precipitated with an equal volume of isopropanol and maintained on ice for 1 hour. The resulting suspension was centrifuged at 13,000rpm for 5 minutes at 4°C and the pellet dried in a

vacuum dessicator before being resuspended in 500ul of TE.

The concentration of DNA in solution was estimated by measuring the optical density (OD) of a 1:100 dilution of the DNA at 260nm in a spectrophotometer (OD of 1=50ug/ml), and purity determined by measuring the OD at 280nm and calculating the 260/280nm ratio (1.8-2.0 is the target range). The plasmid structure was confirmed by restriction enzyme digestion and gel electrophoresis.

#### 3.2.1.8 INTRODUCTION OF EXOGENOUS DNA INTO RODENT CELLS BY ELECTROPORATION

Cultures of the parent fibroblast cell line (208F), or other target cells (M1, T1), were harvested and 0.8ml volumes containing  $5 \times 10^6$  cells/ml PBS were prepared in electroporation cuvettes (Biorad) and maintained on ice. 20ug of circular plasmid DNA was added to the cell suspension, mixed by inversion, and this was kept on ice for 10 minutes prior to electroporation. For each combination of plasmid/cell several electroporation conditions were used, with a fixed capacitance (25uFD) and variable voltage settings (0.4KV, 0.8KV, 1.6KV, 2.4KV), which produced an electrical discharge with 0.5-0.8msec time constants (Biorad Gene Pulser).

The plasmid/cell suspension was held on ice for 10 minutes after electroporation, before addition to 4ml



DMEM/10% HINCS and seeded into small flasks (25cm<sup>2</sup>). These were cultured without selection for 24 hours, followed by selection with geneticin (G418) at 300ug/ml or hygromycin B at 150ug/ml for 20-30 days. Single colonies were isolated and transferred to separate culture flasks to establish monoclonal cell lines.

### 3.2.1.9 DETECTION OF TRANSFECTED DNA IN CELL LINES BY SOUTHERN BLOTTING

#### A) EXTRACTION OF DNA FROM CULTURED CELLS

The technique used was a modification of that described in Maniatis et al (1982). Cells grown in a 150 cm<sup>2</sup> culture flask were harvested and counted as above, and resuspended in 0.1ml PBS. 10ml of lysis buffer (10 mM Tris (hydroxymethyl) aminomethane.HCl (Tris.HCl) pH 7.4, 10mM EDTA, 150mM NaCl) was added. After mixing, sodium dodecyl sulphate (SDS) was added at 0.2% of the final volume, to induce lysis of the cells. RNase A (heat-treated at 10°C for 10 minutes to destroy DNase activity) was added at a final concentration of 50 ug/ml, and incubated at 37°C for 30 minutes. Proteinase K was added at a final concentration of 100 ug/ml and incubated at 48°C for 16 hours.

The sample was extracted in one volume of phenol/chloroform-isoamyl alcohol (24:1) and again by 1 volume chloroform-isoamyl alcohol (24:1). The DNA was precipitated for 16 hours at -20°C after the addition of



one volume of 5M ammonium acetate and 2.5 volumes of absolute ethanol.

The sample was centrifuged at 3000 rpm for 30 minutes at 4°C. The DNA pellet was resuspended in 2 ml of 70% ethanol and centrifuged at 3000 rpm for a further 30 minutes at 4°C. The DNA pellet was dried under vacuum, resuspended in 0.5 ml TE (10mM Tris.HCl pH8, 1mM EDTA) and stored at 4°C.

The concentration and purity of the sample were estimated using an ultra-violet spectrophotometer (Phillips PU8620 series) to measure its optical density (OD). 10 ul of the sample DNA was dissolved in 990 ul TE, and the absorbance of ultra-violet of wavelengths of 260 nm and 280 nm was measured. An  $OD_{260}$  of 1.0 was taken to represent 50ug/ml of DNA in the sample, and an  $OD_{260}:OD_{280}$  ratio of 1.6 - 2.4 was accepted as adequately high purity for further analysis (Maniatis et al, 1982). The DNA was dissolved in TE at 4°C by gentle mixing, and held at this temperature for a minimum of one week prior to use.

#### B) DIGESTION OF CELLULAR DNA BY RESTRICTION ENDONUCLEASE

To 10 ug DNA was added restriction endonuclease (at four times the recommended concentration for cleavage of this amount of DNA, in order to ensure complete digestion) with an appropriate volume of 10x concentrated reaction buffer and deionised distilled water (DDW), to a final

volume of 30 - 40 ul. This was incubated at the appropriate temperature for the restriction endonuclease for 16 hours. An additional 10u of the same restriction endonuclease was added and incubated for a further 3 hours. The reaction was stopped by adding 3 ul of sterile loading buffer (100mM EDTA, 30% sucrose (w/v), 0.25% bromophenol blue [w/v]).

#### C) SEPARATION OF DNA FRAGMENTS BY GEL ELECTROPHORESIS

The digested DNA samples were loaded onto a 0.7% agarose gel in TBE (0.089M Tris.HCl pH8, 0.089M boric acid, 1mM EDTA) and electrophoresed in TBE for approximately 16 hours at 2.6 V/cm, until the bromophenol blue marker had travelled 90% of the length of the gel. The gel was stained in 0.05% ethidium bromide in DDW for 10 minutes, destained in DDW for 15 minutes and photographed under ultra-violet light, to determine the presence of DNA in each track.

#### D) SOUTHERN BLOTTING

The gel was placed in 500 ml of denaturing solution (0.5M NaCl, 1.5M NaOH) for 15 minutes to denature the double-stranded DNA to single-stranded fragments. The DNA fragments within the gel were blotted onto a nylon filter (Genescreen Plus; DuPont) by capillary blotting (Southern, 1975). The gel was placed on top of a wick of filter paper (2 sheets Whatman No.6, 3mm paper) supported on a glass plate, with the ends of the wick

submerged in 500 ml of 0.5M NaOH/ 1.5M NaCl solution. A nylon filter, cut to the same size as the gel, was placed carefully on top of the gel, excluding air bubbles. Above this was placed four sheets of filter paper (Whatman No.6) cut to the size of the gel, a 30 cm pile of paper towels, and a 1 kg weight. Capillary blotting occurred over 24-48 hours, changing the saturated paper towels as necessary.

The filter was retrieved and neutralised in buffer (0.5M Tris.HCl pH7.4, 3M NaCl) for 30 - 60 minutes. It was dried at room temperature to anchor the DNA fragments, and stored in a sealed polythene bag at room temperature.

#### E) PREPARATION AND LABELLING OF HYBRIDISATION PROBES

Filters were probed for the presence of transfected DNA. Hybridisation probes included myc and ras oncogene DNA derived from the transfected plasmids, by cleavage with appropriate restriction endonucleases. The cleaved DNA fragments from these restriction reactions were electrophoresed in low melting temperature agarose, and the DNA fragments to be used as hybridisation probes were cut out of the gel. These probes were extracted once in phenol/chloroform - isoamyl alcohol (25:24:1) and precipitated for 60 minutes at -20°C after addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol.

The probes were radioactively labelled by the random

primer oligolabelling method (Klenow fragments, random hexamer primers and reaction buffer from Pharmacia) using a procedure based on that of Feinberg and Vogelstein (1983 and 1984). To a volume containing 100 ug of probe DNA was added DDW, 10 ul of nucleotide and buffer solution (dATP, dGTP and dTTP), 2ul bovine serum albumin (BSA; DNase and RNase free), 2ul purified cloned Klenow fragment of DNA polymerase 1 (E. Coli), and 5ul <sup>32</sup>P-labelled d-CTP (50 uCi; Amersham Intl.). This was incubated at 20°C for 16 hours. 2 ul of "stop" buffer (d-CTP, EDTA, SDS, NaCl, and buffer) and 30 ul of DDW were added. The sample was passed down a Sephadex column (Nick Column; DuPont) to remove unincorporated nucleotides, and the specific activity of a 5 ul sample in 4 ml of scintillant was measured using a scintillation counter (LKB Rackbeta). The probes were used immediately.

#### F) PREHYBRIDISATION, HYBRIDISATION AND WASHING

i). Prehybridisation: 30 ml of prehybridisation solution was prepared, containing 6 x SSC (sodium citrate pH 7, sodium chloride), 1% SDS (w/v; pH7.2), 10% dextran sulphate and 3 mg sonicated salmon sperm DNA (heated to 100°C for 10 minutes and snap-cooled on ice to denature the DNA) in DDW, and incubated at 65°C for 30 minutes to remove air bubbles. This solution was added to a polythene bag (Hybaid system) containing the nylon filter to be probed, the bag was sealed, and incubated at 65°C

for 4 - 6 hours.

ii). Hybridisation: 10 - 20 ml of the prehybridisation solution was removed from the bag and to this was added the radiolabelled probe plus sonicated salmon sperm DNA (heat denatured at 100°C for 10 minutes and snap-cooled on ice) at a final concentration of 25mg/ml. The hybridisation solution plus probe was inserted into the bag, sealed and inverted several times to ensure uniform bathing of the filter. This was incubated at 65°C for 16 hours.

iii). Washing: The polythene bag containing the nylon filter was drained of hybridisation solution and the filter was washed three times, once in 2 x SSC, and twice in 2 x SSC / 1% SDS using the Hybaid apparatus (Hybaid). The filter was removed from the bag and washed in 0.1 x SSC for 30 minutes. The filter was sealed in a polythene bag without allowing it to dry to allow re-probing if necessary.

#### G) AUTORADIOGRAPHY

The sealed bag containing the nylon filter was placed next to an Fuji RX X-ray photographic film in an X-ray cassette (Kodak), and left at -70°C. The film was developed in D19 developer (Kodak) for 5 minutes, washed briefly in water (10 - 15 seconds), fixed in FX40 fixative (Kodak) for 8 minutes at 20°C, washed in tap water for 30 minutes and dried.

#### 3.2.1.10 DETECTION OF TRANSFECTED DNA IN CELLS BY POLYMERASE CHAIN REACTION

Protocols used for the preparation of cells and amplification of defined target DNA sequences were identical to those described in section 2.2. The HPV type-specific primers, previously described and validated (section 2.3), were used for HPV DNA amplification. Primers specific for c-Ha-ras, c-myc and human beta actin were designed and applied to the oncogene transfected cell lines (section 3.5).

#### 3.2.1.11 PREPARATION OF RNA FROM CELL CULTURES

$5 \times 10^7$  cells were cultured in a 75cm<sup>2</sup> flask, the medium was removed and the cells rinsed twice in ice-cold PBS. 4ml ice-cold ULC (6M Urea, 3M Lithium Chloride) was added to the flask, which was maintained at 4°C for 1 hour, to lyse the cells and solubilise RNA. This solution was removed to a fresh sterile tube and the flask rinsed out with a further 4ml ULC, which was also added to the sterile tube. This solution was strictly kept at 4°C, sonicated on ice with 4 bursts of 15 seconds (MSE cell disruptor, set at 10mA), and maintained at 4°C for 18 hours. This solution was transferred to a sterile Corex tube and centrifuged at 10,000rpm for 30 minutes at 4°C. The supernatant was discarded and pellet resuspended in 0.5ml 10mM Tris (pH 7.4), 10mM EDTA, 0.1% SDS at room

temperature. In sterile Eppendorf tubes this solution was extracted once in an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) and again in an equal volume of chloroform:isoamyl alcohol alone. A tenth volume of 5M Ammonium Acetate and one volume of absolute ethanol were added and the solution stored at  $-20^{\circ}\text{C}$ . The concentration of RNA was measured using a 1:100 dilution TE to determine the optical density at 260nm ( $\text{OD}_{260}=40\mu\text{g/ml}$  for s/s RNA;  $\text{OD } 260/280$  ratio of 2.0 = ideal purity). For recovery of RNA, the appropriate volume was removed from  $-20^{\circ}\text{C}$ , a further 1 volume of absolute ethanol was added and the solution maintained at  $-70^{\circ}\text{C}$  for 18 hours. This was centrifuged at 13,000rpm for 20 minutes at  $4^{\circ}\text{C}$ , the pellet was dried and resuspended in the appropriate volume of TE.

#### 3.2.1.12 ANALYSIS OF CELLULAR RNA

##### A) FORMALDEHYDE-AGAROSE GEL ELECTROPHORESIS OF RNA

A 1% agarose solution in MSE was prepared containing formaldehyde (1 volume 12.3M formaldehyde to 4.6 volumes agarose solution, added after cooling below  $60^{\circ}\text{C}$ ). 10 x MSE running buffer (0.2M morpholinopropanesulfonic acid [pH 7.0], 50mM sodium acetate, 10mM EDTA [pH 8.0]) for RNA gel electrophoresis was prepared. RNA samples of 10ug in 4.5ul were prepared in sterile tubes with 2ul of 10 x MSE, 3.5ul 12.3M formaldehyde and 10ul formamide, and incubated at  $55^{\circ}\text{C}$  for 15 minutes to denature any RNA

secondary structure. To this was added 2ul sterile RNA loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and these samples were loaded into the gel for electrophoresis as above.

#### B) DOT BLOT HYBRIDISATION OF RNA

10ug samples of cellular RNA were denatured as above in 15 x SSC and transferred to nitrocellulose filters using Hybridot apparatus following the manufacturer's instructions, and the filters baked at 80°C in a vacuum for 1 hour. These were pre-hybridised, hybridised and washed using the same conditions as for Southern blot hybridisation.

#### C) REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS OF RNA

To detect the expression of specific genes, RNA prepared from cells was amplified using a two stage procedure. First, RNA was copied to cDNA by reverse transcription. Second, cDNA was amplified using the polymerase chain reaction (PCR). Strict PCR precautions against contamination by extraneous sequences (see chapter 2) were observed throughout including use of autoclaved tubes, gloves, preparation in a laminar air flow Class II biological safety cabinet, and maintenance of all solutions on ice during preparation stages.

The reverse transcriptase (rT) reaction consisted of the



following reagents in a final volume of 20  $\mu$ l: 1 X PCR buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3 at room temperature], 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin), 1 mM each dNTP, 1 unit/ $\mu$ l of RNasin, 100 pmol downstream PCR primer (primer 2), 10  $\mu$ g of RNA (in 10  $\mu$ l TE), and 200 units of Moloney Murine Leukaemia Virus (MoMuLV) reverse transcriptase enzyme (Gibco-BRL). The RNA was heated to 90° for 5 minutes and snap-frozen on ice, prior to addition to the rT reaction solution, to denature RNA secondary structure. The rT reaction solution was incubated for 10 minutes at 20°C, followed by 45 minutes at 42°C, and finally 10 minutes at 95°C with snap cooling on ice. The final 95°C heat treatment denatured the RNA-cDNA hybrids and inactivated the reverse transcriptase.

The second stage of PCR amplification consisted of adding to the heat treated rT reaction solution, 80  $\mu$ l of 1 X PCR buffer, 100 pmol of upstream PCR primer (primer 1), 2.5 U of Taq Polymerase (Cetus-Perkin-Elmer), and 200  $\mu$ M of each dNTP. This was mixed, and a layer of 100  $\mu$ l of mineral oil was added to the top of the PCR solution, to prevent evaporation of liquid. Thermal cycling on an automated heating block (Hybaid), consisted of 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 2 minutes, and DNA synthesis at 72°C for 3 minutes. The initial cycle had a 94°C denaturation step of 1.5 minutes duration, and the final cycle had a 72°C DNA synthesis step lasting for 10 minutes. 30  $\mu$ l of the reaction product was electrophoresed on a 2% agarose gel

(3:1 Nusieve GTG:Seakem agarose) containing 2 ug/ml ethidium bromide, and this was visualised under UV light.

### 3.2.2 CELL BIOLOGY TECHNIQUES

#### 3.2.2.1 FIBROBLAST CELL CULTURE

Sterile reagents, equipment and technique were employed. The fibroblast cell lines were maintained as monolayer cultures attached to plastic tissue culture compatible flasks (Costar). Culture medium used was the Glasgow modification of minimum Eagle's medium (GMEM: Northumbria Biologicals), supplemented with L-glutamine (228mg), sodium bicarbonate (0.01M) and 10% heat-inactivated neonatal calves' serum (HINCS: GIBCO) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

To harvest a culture monolayer, the cells were washed briefly (5-30 seconds) in 0.02% ethylene diamine tetraacetate (EDTA) in phosphate buffered saline (PBS). The EDTA was replaced by a solution of 0.1% trypsin in PBS and incubated at 37°C until the monolayer had disaggregated into a single cell suspension. This was transferred to a conical-bottomed universal container, and an equal volume of GMEM/10%HINCS added to neutralise the trypsin. The cells were centrifuged at 1000 rpm for 10 minutes at 20°C, and resuspended in PBS. A sample of cells was counted prior to further use.

Primary cultures of rat fibroblasts were also prepared for comparison with immortalised 208F cells and experimentally transformed cells. The lungs were removed from a freshly-killed 6 week old female Sprague-Dawley

rat and dissected into 0.25 - 1 mm fragments. These fragments were digested in a sterile solution of 0.25% trypsin, 2.5 ug/ml amphotericin and 100 ug/ml kanamycin in phosphate buffered saline (PBS) at 37°C for 60 minutes, mixing frequently. The sample was centrifuged at 1000 rpm for 10 minutes at 20°C. The supernatant (including any large fragments not in the pellet) was discarded. The pellet, containing single cells and small fragments, was resuspended in 20 ml GMEM/10% HINCS and filtered through nylon wool to remove any large clusters of cells. The remaining cells, in single cell suspension, were prepared for flow cytometry.

#### 3.2.2.2 PREPARATION OF CELLS FOR TRANSFECTION BY ELECTROPORATION

Parent cells for transfection, mostly 208F Fischer rat fibroblasts, but also M1 and T1 fibroblasts in some experiments, were harvested while growing in log phase (50-80% confluence). They were washed twice in ice cold sterile PBS, counted and resuspended at a concentration of  $5 \cdot 10^6$  in 0.8ml PBS, prior to addition of 20ug of plasmid DNA and electroporation (see section 3.2.1.8).

#### 3.2.2.3 FREEZING AND THAWING OF CELLS

Following transfection and cloning, cell lines were stored in the vapour above liquid nitrogen ( $N_2$ ) at

-196°C, in 10% dimethyl sulphoxide (DMSO) and 50% HINCS. Cells were thawed rapidly at 37°C (2-3 minutes) and immediately mixed with 20ml of GMEM to dilute the toxic effects of DMSO at room temperature or above. The cells were centrifuged at 1000rpm for 10 minutes at 20°C, washed in PBS, resuspended in 20ml of GMEM/ 10%HINCS and seeded into a culture flask. Cells were initially grown in a humidified 5% CO<sub>2</sub> incubator at 37°C, but after passage, the pH of the medium was adjusted to 7.4 with 5% CO<sub>2</sub> and the cells were grown in sealed flasks at 37°C.

An aliquot of frozen cells from each cell line was subsequently frozen in liquid N<sub>2</sub> to maintain a frozen stock of cells. Cells were harvested, washed in PBS and counted, centrifuged at 1000rpm for 10 minutes at 20°C, and resuspended in a volume of cryoprotectant (10% DMSO, 50% HINCS) at a final concentration of 2 - 5 x 10<sup>6</sup> cells per ml. The cell suspensions were frozen slowly (approx. 1°C per minute) by placing them at -70°C for 16 hours. The cells were transferred to liquid N<sub>2</sub> for long-term storage.

#### 3.2.2.4 QUANTITATION OF CELL CONCENTRATION

The cell concentration was determined using the Neubauer improved haemocytometer. Two separate samples were taken from a single cell suspension, four independent counts were made of the cell number, and the mean calculated.

### 3.2.2.5 ASSESSMENT OF MORPHOLOGY OF APOPTOSIS

#### A) ELECTRON MICROSCOPY

Two specimens were collected: (1) cellular bodies released into the medium above a growing culture of cells, and (2) monolayer cells attached to the substratum. These were prepared for electron microscopy by standard methods. The cellular bodies were washed in PBS, and centrifuged at 1000 rpm for 10 minutes at 20°C. The pellet was resuspended and fixed in 3% glutaraldehyde in sodium cacodylate for four hours, rinsed in sodium cacodylate buffer and fixed in 1% sodium tetroxide. The sample was dehydrated through increasing grades to 100% ethanol. The sample was impregnated through grades of araldite, to 100% araldite; after 1 hour, the araldite was polymerised at 56°C. Representative sections were cut, 50 - 60 nm thick, and stained with uranyl acetate and lead citrate. The specimen grids were viewed and representative fields photographed.

#### B) FLUORESCENCE MICROSCOPY

Samples of the cellular bodies released into the medium above cells attached to the flask substratum were stained with equal volumes of 10ug/ml acridine orange in PBS and viewed under UV light.

### 3.2.2.6 ASSAY OF ENDOGENOUS ENDONUCLEASE ACTIVITY

Monolayers of near-confluent cells growing in 175cm<sup>2</sup> flasks were harvested, washed in PBS and the total numbers counted. The cells were centrifuged at 1000 rpm for 10 minutes at 20°C, and resuspended in 3 ml of buffer A (150mM NaCl, 1.5mM CaCl<sub>2</sub>, 10mM Tris.HCl pH7.4, 3% [v/v] glycerol, autoclaved prior to use) at 4°C; all succeeding reagents and apparatus were pre-cooled to 4°C. 3ml of freshly-prepared buffer B (90% [v/v] buffer A, and 10% [v/v] Nonidet P40) was added to induce lysis of the cells. After 15 minutes the cell lysate was layered on top of 3ml of buffer C (25% [v/v] glycerol, 10mM Tris.HCl pH7.4, 1.5mM CaCl<sub>2</sub>, autoclaved prior to use) in a Corex test tube, and centrifuged at 600 rpm for 10 minutes at 4°C. The supernatant was discarded and the white pellet containing the nuclei retained. 200 ul of buffer A was added, the pellet resuspended, and the volume accurately determined. A further volume of buffer A was added and agitated gently. Samples of the processed nuclei were used in an overnight incubation to test for calcium- and magnesium-sensitive endogenous endonuclease activity capable of digesting the nuclear chromatin in the presence or absence of divalent cations. To demonstrate that chromatin cleavage was enzymic, zinc ions were added at 2mM to inhibit the reaction.

Three 100 ul samples were taken from each preparation of nuclei. These were incubated at 37°C for 16 hours after

adding  $\text{CaCl}_2$  to give a final concentration of 1.5mM  $\text{Ca}^{2+}$  to the first,  $\text{MgCl}_2$  plus  $\text{CaCl}_2$  to give a final concentration of 1.5 mM of each of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  to the second, and 100mM EDTA and 100mM EGTA (chelators of divalent cations) to the third, as a control.

After the overnight incubation at 37°C, 30 ul of 0.2M EDTA was added to each of the three test cell samples and they were incubated at 4°C for 30 minutes. All samples were centrifuged at 1300 rpm for 5 minutes at 20°C. DNA was precipitated by the addition of 30 ul 5M NaCl, and 600 ul absolute ethanol at -20°C for 16 hours.

The samples were centrifuged at 1300 rpm for 15 minutes at 4°C and the supernatants discarded. DNA fragments in the pellets were resuspended in 100 ul TE, and 5 ul 10% [v/v] SDS was added. The sample was extracted through an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). 25 ul of each sample was removed and to this was added 5 ul DNA loading buffer (100mM EDTA, 30% [w/v] sucrose, 0.25% [w/v] bromophenol blue) and the sample electrophoresed on a 1% agarose gel in TBE, also containing 0.05% ethidium bromide. The gel was photographed under ultra-violet light. This assay ascertained the presence or absence of a "chromatin ladder" of DNA fragments signifying endonuclease activity typical of apoptosis. The DNA content of bands in the gel tracks was assessed by laser densitometry (LKB Ultrascan XL Enhanced Laser Densitometer).



### 3.2.2.7 RATE OF CELL POPULATION EXPANSION

Nine tissue culture compatible Petri dishes (Falcon; Becton Dickinson) were seeded with  $3.5 \times 10^5$  cells in 5 ml GMEM/10% HINCS for each cell line. The dishes were incubated at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere. At 24, 48, and 72 hours the cells were counted as described above, using three of the dishes at each time point and calculating the mean value.

The rates of expansion of the different cell lines were compared by calculating the proportional increase in terms of the ratio of cells at 48 and 24 hours ( $N_{48}/N_{24}$ : where N is the number of cells at each time point). This choice of time points was designed to allow for three potential problems. (1) Differences in seeding efficiency between the cell lines were minimised. (2) The cells were allowed to enter the phase of maximal growth (unlimited by growth factors in serum or nutrients in the medium). (3) The cells had not reached confluence by the end of the test period. The figure of  $3.5 \times 10^5$  cells to be seeded initially was arbitrarily chosen to give a large enough sample of cells for accuracy of counting, and also so that the cells were not significantly confluent after 72 hours growth in vitro. However, some variation in the expansion rate between 48 and 72 hours could be caused by localised areas of cells in culture becoming confluent and undergoing contact inhibition of growth (for some cell lines), or by depletion of growth factors or

essential nutrients from the medium. Thus, the 24 to 48 hour period excluded any such bias.

There were two reasons for using, as a measure population expansion, the proportional increase ( $N_{48}/N_{24}$ ) rather than the linear increase ( $N_{48} - N_{24}$ ), as measured by the gradient of the expansion curves. First, although the rate of population expansion did not conform exactly to a simple mathematical equation, the shape of the population expansion curve approximated more closely to an exponential pattern of growth rather than a linear pattern (see figure 3.16). Second, the gradient was more likely to be significantly affected by small differences in cell number at 24 hours, as may have been caused by different seeding efficiencies of the cell lines, or minor errors in the numbers of cells seeded.

#### 3.2.2.8 CELL PROLIFERATION INDEX

An EPICS (Electronically Programmable Individual Cell Sorter) flow cytometer (Coulter Electronics; EPICS CS system) was used for cell cycle analysis by determination of the proportion of cells in each phase of the cell cycle according to DNA content. The method used cells growing in maximal growth phase in culture. All samples for flow cytometric study were prepared by the method of Vindelov et al (1983a); nuclear preparations were obtained and stained with propidium iodide (Fluka), a fluorescent dye which binds DNA with a stoichiometric

relationship, and the fluorescence was measured on the flow cytometer.

The cells to be analysed were passaged into 75 cm<sup>2</sup> culture flasks in a ratio of approximately 1:4 from confluence 48 hours before the analysis was carried out. This ensured that the cells were growing in maximal growth phase at 50 - 70 % confluence, ensuring the cell population contained actively proliferating cells, as confluent monolayers demonstrate decreased growth rates (Martz and Steinberg, 1973). The medium was removed from each flask (retained at 4°C for subsequent analysis of the cellular bodies), and replaced with 20ml GMEM/10% HINCS, so that cells were not growth limited by utilisation of the essential nutrients. Flasks were incubated at 37°C for 4 hours.

The cells were harvested, washed in PBS and counted, and centrifuged at 1000 rpm for 10 minutes at 4°C. 10<sup>6</sup> cells were resuspended in 100 ul citrate buffer (250mM sucrose, 40mM trisodium citrate.2H<sub>2</sub>O pH7.6, 5% [v/v] DMSO). To each cell sample was added 20 ul of chicken red blood cells (approximately 10<sup>6</sup> cells) in DMSO, to act as an internal standard for each trial (Vindelov et al, 1983b). 450 ul of solution A (3mg trypsin in 100ml stock solution [3.4mM trisodium citrate.2H<sub>2</sub>O, 0.1% [v/v] Nonidet P40, 1.5mM spermine tetrachloride. 0.5mM Tris.HCl pH7.6]) was added, and the sample incubated at 20°C for 10 minutes. 325 ul of solution B (50mg trypsin inhibitor, 10mg

RNAse A [heat treated at 100°C for 10 minutes to eliminate DNase activity] in 100ml of stock solution) was added, and the sample incubated for 10 minutes at 20°C. 250 ul of solution C (41.6mg propidium iodide and 100mg spermine tetrachloride in 100ml stock solution) at 4°C was added, and the sample incubated on ice for 10 minutes. The cells were fully resuspended by reflux using a fine needle to disaggregate clumps of cells and produce a single cell suspension. The processed cell samples were analysed within 30 - 60 minutes of preparation, although they were known to be stable for up to three hours (Vindelov et al, 1983a).

The flow cytometer passes a stream of single cell nuclei through a laser beam (known to excite the fluorescence of the DNA-bound propidium iodide) and records the amount of fluorescence produced by each nucleus. Data is stored on computer disc, and can be viewed as a two parameter histogram of red fluorescence (DNA content) versus total number of cells, as shown in figure 3.19. The results were subsequently analysed using two different computer software programs, PARA 1 and SFITS (Coulter Electronics). These programs use different mathematical models to estimate the proportion of cells in each phase of the cell cycle.

The cell cycle profile was used to compare the cell cycle parameters for different cell lines, to give a measure of the proliferative capacity of the cells following 48

hours growth. The proportion of cells in S plus G2/M phases of the cell cycle was defined as the proliferative index in vitro.

Cell cycle profiles were assumed to be similar for the various cell lines, in terms of the lengths of the three separately determined phases or phase combinations (G0/G1, S and G2/M), and that the distribution of the cells within S phase is similar. The validity of these assumptions is supported by the derivation of all transfectants from the same parent cell line (208F), and the lack of apparent differences in the cell cycle profiles observed. Cell samples were tested in maximal growth phase (40 - 70% confluence), when growth was not limited by a lack of nutrients or growth factors. This provided a measure of proliferation during maximal growth phase of the cell population.

The computer analysis of the cell cycle relied on analysis of the total DNA content of the cells and used mathematical models to determine the proportions of cells in each phase of the cell cycle (Baisch et al, 1982). This method of analysis of the proportion of cycling cells may have been inaccurate if the cells became blocked in S phase, if the cells became synchronised in their passage through the cell cycle, or if a subpopulation of any cell line had evolved a different DNA ploidy compared with the majority of the cells. The initial use of two different computer programs (PARA 1

and SFITS, using different mathematical models) on eleven of the cell lines produced similar data. The homogeneous samples of asynchronously growing cells allowed minimisation of these differences. No separate aneuploid peaks or unusual peaks in the S phase area of the DNA histogram were observed.

One factor which may theoretically alter the cell cycle profile of some of the cell lines is the proportion of normal cells released into the growth medium. Cells tend to round up during mitosis, and some may lose contact with the culture flask substratum. For some of the T24-ras transfected cell lines, 10% - 50% of the released cellular bodies had the morphology of normal cells, and it is possible that erroneously low proliferative indices were measured using monolayer cells for the assay, due to the loss of some cells in the G2/M phases of the cell cycle. However, the total number of viable cells released into the medium was usually fairly small (about 3% - 8% of the total number of attached cells) for those cell lines with high proportions of viable released cellular bodies, indicating that this potential source of error was numerically small.

#### 3.2.2.9 RATE OF CELL DEATH BY APOPTOSIS

The medium which was removed from the monolayer cells growing for flow cytometric analysis was centrifuged at 1000 rpm for 10 minutes at 20°C, and the cellular bodies

resuspended in 0.5 ml PBS. This sample was counted for the total number of cellular bodies using an improved Neubauer haemocytometer. 0.2 ml of this sample was mixed with 0.2 ml of acridine orange (0.5 mg/ml) (a DNA and RNA binding dye) on a glass slide, and viewed under ultra-violet light. 100 - 200 cellular bodies were counted, quantifying the proportion of apoptotic bodies and non-apoptotic cells, differentiated by the characteristic morphological differences between apoptotic and normal cells.

The apoptotic index was calculated as a measure of production of apoptotic bodies in 48 hours, taking into account the number of surviving cells, according to the following equation:

$$\text{Apoptotic Index (AI)} = \frac{\%A \times B \times 100}{(\%NA \times B) + C}$$

B = Total number of cellular bodies released into the medium above a growing culture of cells in 48 hours.

%A = Proportion of B which consists of apoptotic cells.

%NA = Proportion of B which consists of non-apoptotic cells.

C = Total number of surviving monolayer cells after 48 hours.



This experimental technique was developed for this study from previously used methods (A.Wyllie, personal communication). A number of points arise regarding its design.

(i) The method used to measure the rate of apoptosis utilised the observation that one of the earliest events in apoptosis is the loss of cell-cell contact producing detachment of apoptotic cells from their viable neighbours (Kerr et al, 1972; Wyllie et al, 1981; Wyllie, 1985), and also from the culture substratum. The vast majority of these cells subsequently float freely in the growth medium, with only occasional phagocytosis of apoptotic cells by adjacent viable cells. Those cell lines with high apoptotic rates (c-myc transfectants) showed 70% - 90% of the released cellular bodies were apoptotic in nature. For some of the cell lines with low apoptotic rates (T24-ras transfectants and MT7) the proportion of apoptotic cells in the medium was frequently as low as 10 - 20% (although the total number of released cells and bodies was always considerably smaller than the number of monolayer cells). This effect was taken into account in the design of the equation for calculation of the apoptotic index.

(ii) Depletion of serum growth factors was known to increase the rate of apoptosis in some cell lines, (eg M1 A.Wyllie, personal communication). This potential source of error was avoided by collecting released cell bodies



over the relatively short period of 48 hours, before cell growth had significantly depleted growth factors. Low densities of cells, never reaching more than 70% also limited growth factor depletion.

(iii) Culture medium was removed from the assay flask of cells four hours before the monolayer cells were harvested, and this medium was used to analyse the released cellular bodies. The same samples of monolayer cells were used to measure cell proliferation.

(iv) The method of quantification of the total number of released cellular bodies by phase-contrast microscopy was designed to take into account the small size of some of the released apoptotic bodies. A cut-off size threshold was used for each count, based on the criteria that cellular bodies were membrane-bound (rejecting large debris), contain nuclear material (rejecting non-cellular matter), and bodies below a certain size (25% of the diameter of viable cells) were not counted. Considerable care was taken to use the same size cut-off point between different cell lines, since some lines (those with higher apoptotic indices) tended to have more small cellular debris within the medium. On several occasions, and on the first count in any experiment, counting of the same cell population was repeated to determine reproducibility; this was found in almost all cases to be greater than 95%.

(v) The quantitation of apoptotic versus non-apoptotic

(viable) cellular bodies was determined by fluorescence microscopy after staining with acridine orange, based on the characteristic morphological appearances of apoptotic bodies. In a small proportion of cases it was not possible to classify some cellular bodies due to ambiguous morphological appearances, poor staining or overlapping of cells; these bodies were categorised as neither apoptotic nor viable cells, and this category never exceeded 5% of the total number of cells counted.

(vi) The apoptotic index was calculated based on the number of monolayer cells after 52 hours growth in a flask. A possible future refinement of the technique which may improve the accuracy of the apoptotic index would be to determine the exact number of cells seeded into the flask, allowing for possible differences of seeding efficiencies and expansion rates of the cell lines, in order that similar numbers of cells would be present in each flask after 52 hours growth.

#### 3.2.2.10 ANALYSIS OF TUMOUR GROWTH PARAMETERS IN VIVO

All transfected fibroblast cell lines were analysed for in vivo tumour growth, including the parent immortalised rat fibroblast cell line (208F), ten oncogene transfectants and four HPV/oncogene transfectants.  $10^7$  cells from each cell line were obtained from 150cm<sup>2</sup> culture flasks grown to near 100% confluence. The cells were harvested with 0.02% EDTA and 0.1% trypsin, washed

and resuspended in PBS at a concentration of  $10^8$  cells per ml. The assay used between six and twelve female mice (CBA), previously rendered immune suppressed by thymectomy, total body x-irradiation of 750 rads and treatment with cytosine arabinoside (supplied by Dr L Foster, prepared using the method described by Wyllie et al, 1987).  $10^7$  cells, suspended in 0.1 ml of PBS, from each of the cell lines were injected subcutaneously into the left groin of each mouse. Most animals were killed after 12 days, and autopsies were performed.

The size of the primary tumour was measured in three dimensions, and multiplied to calculate a tumour "box" volume. Local tumour spread and distant metastasis were also assessed. A small number of animals were killed earlier (9-11 days) if signs of terminal illness were evident (this only affected T1 and MT7). In order to compare the relative rates of tumour growth in vivo, the tumour "box" volume attained at 12 days was taken as an approximate measure. As some experiments were terminated before 12 days, the tumour volumes were adjusted to take this into account, by standardizing to 12 days. This adjustment assumed linear growth over the final 1-3 days of the 12 day period, almost certainly underestimating the final tumour size generated by T1 and MT7 cells, which produced some of the largest tumours. Errors introduced by this simple calculation were more likely to reduce, rather than increase, the chances of finding statistically significant differences when compared with

the smaller tumours produced by the parent and c-myc transfected cell lines.

Blocks of tumour were taken and analysed histologically for mitosis and cell death. The number of mitotic and apoptotic figures per ten high power (x400) fields were determined. The criteria set out by Baak (1990) were used for identification of mitotic figures: absence of nuclear membrane, absence of clear zone in nuclear centre, presence of hairy rather than triangular or jagged chromosomal projections, relative basophilia of surrounding cytoplasm instead of eosinophilia, and omission of doubtful structures. Counting mitotic figures is a well established histological method (Silverberg, 1976; Scully, 1976; Norris, 1976) that can generate reliable comparative data (Ellis and Whitehead, 1981), particularly under these ideal experimental conditions where there was no fixation delay (Donhuijsen et al, 1990).

Apoptotic figures were identified using criteria set out by Kerr et al (1972) and Wyllie et al (1980): condensation of nuclear chromatin into densely staining crescentic caps at the periphery of the nucleus, or more commonly chromatin condensation into variably sized spheres which are often multiple, eosinophilia of surrounding cytoplasm, clusters of eosinophilic bodies of variable sizes containing variable numbers of condensed chromatin spheres, often within an apparent space in the

tissue rather than closely abutting onto adjacent cells, and omission of doubtful structures. Tumour necrosis was identified as confluent zones of eosinophilic material often containing cell ghosts, and was assessed semi-quantitatively using an arbitrary scale of 0-4.

Mitotic and apoptotic counts made from tissue sections vary with three parameters. (1) The frequency of occurrence of the event (mitosis or apoptosis). (2) The duration of histological observability of the event. (3) The proportion of the population capable of undergoing each event. In this fibroblast series, mostly homogenous monoclonal cell lines were used for comparison, and these were derived from the same parent fibroblast line. Both of these elements in the design of the system should minimise possible sources of error due to variations in the second and third parameters described above. Finally, the calculation of the ratios of mitosis:apoptosis (M/A), or vice versa (A/M), was designed to cancel out effects due to differences in high power field samples, including cell size and density. Thus, the M/A or A/M ratios were designed to be single indices that reflected cell turnover in terms of the relative frequencies of mitosis and apoptosis, allowing more accurate comparison of lines with different cell sizes.

### 3.3 PLASMID STRUCTURE AND CONSTRUCTION

#### 3.3.1 EXISTING PLASMIDS

#### 3.3.2 CONSTRUCTION OF NEW PLASMIDS

The fibroblast cell lines under study were derived from the 208F rat fibroblast cell line (Quade, 1979) by transfection with expression plasmids containing c-myc (pMCGM1 or pHRMCGM1), mutated c-Haras1 (pH05T1 or pHRH05T1, both containing the T24-ras oncogene), and HPV 6b, 11, 16 and 18 (partial viral genomes containing the URR, the two transforming genes E6 and E7, and most of the other early genes E1, E2, E4, and E5, but only part or none of the two late genes L2 and L1), subcloned into the pJ40 expression vector.

#### 3.3.1 EXISTING PLASMIDS

The structures of pH05T1, pMCGM1 and the pJ40.HPV plasmids are shown in figures 3.1-3.3.

pH05T1 was constructed by Spandidos and Wilkie (1984). The T24 human c-Ha-ras-1 gene (T24-ras contains a point mutation at codon 12 - encoding valine instead of glycine) in a 6.6kb DNA fragment (Santos et al, 1982; Reddy et al, 1982; Capon et al, 1983). This fragment was inserted into the Homer 5 expression vector at the Bam H1 cleavage site, forming a 15.3kb plasmid. The Homer 5 plasmid contains a powerful constitutive

promoter/enhancer element derived from the genome of Simian Virus 40 (SV40), plus the aminoglycoside phosphohydrolase (aph or neo) gene which confers resistance to geneticin (G418) (Colbere-Garapin et al, 1981). G418 is a powerful aminoglycoside antibiotic which is lethally toxic to mammalian cells in culture at doses of 100 to 400ug/ml.

pMCGM1 was constructed (Spandidos and Wilkie, 1984) by inserting a 2.9kb DNA fragment containing the aph gene linked to the Moloney Virus long terminal repeat sequence (MoLTR: known to contain a powerful constitutive promoter/enhancer element) into the Eco R1 cleavage site of plasmid pMC41C1 (Watson et al, 1983; Battey et al, 1983), which contains the human c-myc proto-oncogene, to form a 15.6 kb plasmid.

pJ40.HPV plasmids were constructed by Storey et al (1988) by insertion into the multiple cloning site of the parent vector pJ40mega, of fragments of HPV genomes, containing all the early genes and most of the upstream regulatory region, cleaved with appropriate restriction enzymes (figure 3.3).

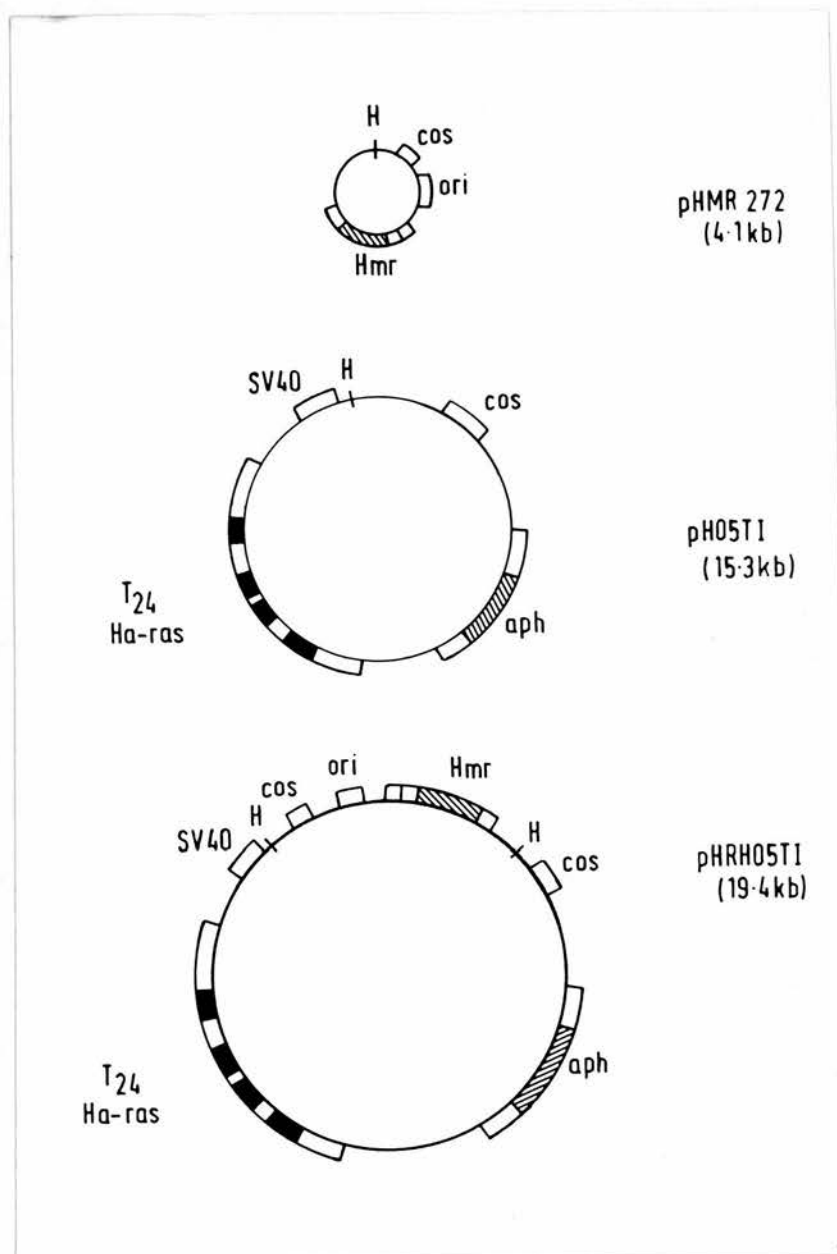


Figure 3.1

Plasmid maps of pHMR272, containing the hygromycin resistance (Hmr) gene, and pH05T1 containing the T24-ras gene with 4 exons, and the geneticin resistance gene (aph). These were both cleaved at the unique HindIII sites and ligated to produce pHRH05T1.



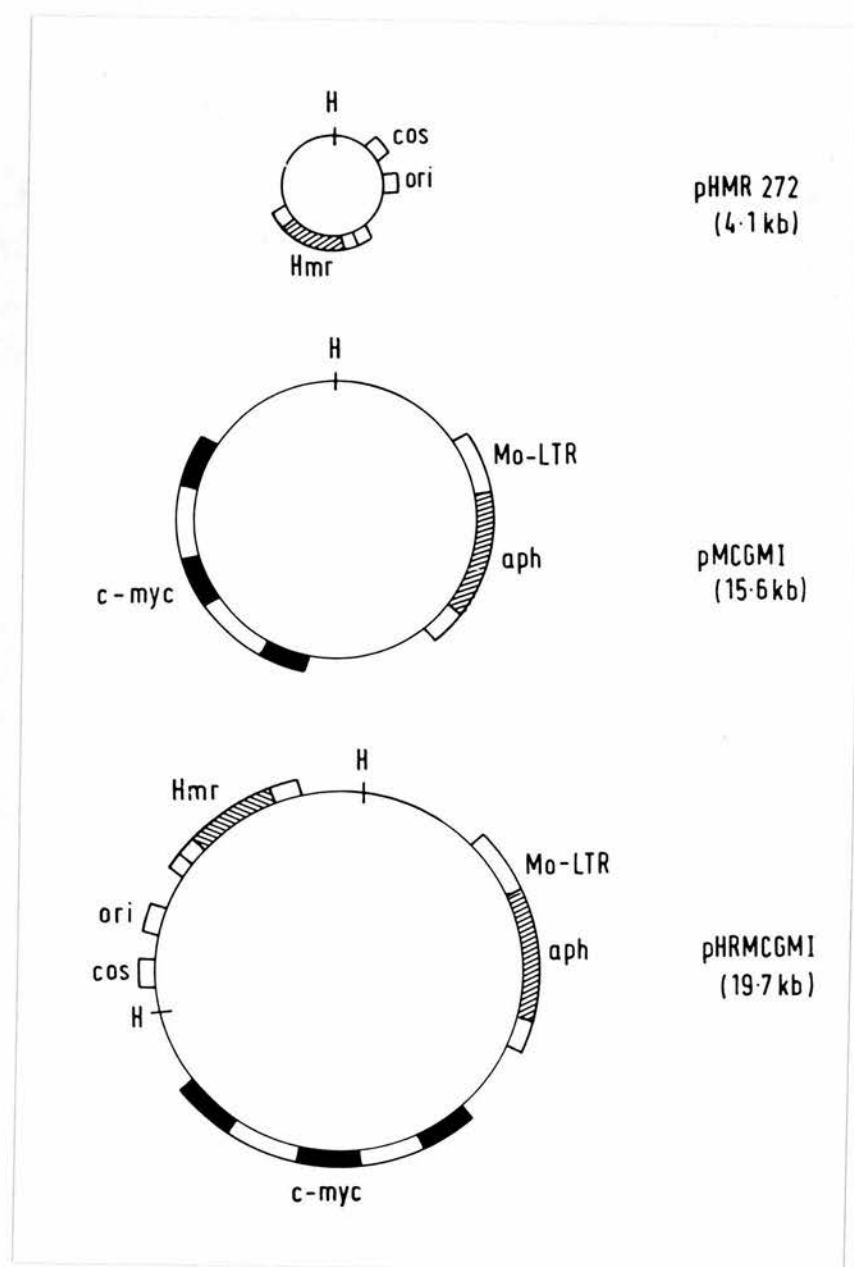


Figure 3.2

Plasmid maps of pHMR272, containing the hygromycin resistance gene (*Hmr*), and pMCGM1 containing the *c-myc* oncogene with 3 exons, and the geneticin resistance gene (*aph*). These were cleaved at their unique *Hind*III sites and ligated to form pHRMCGM1.

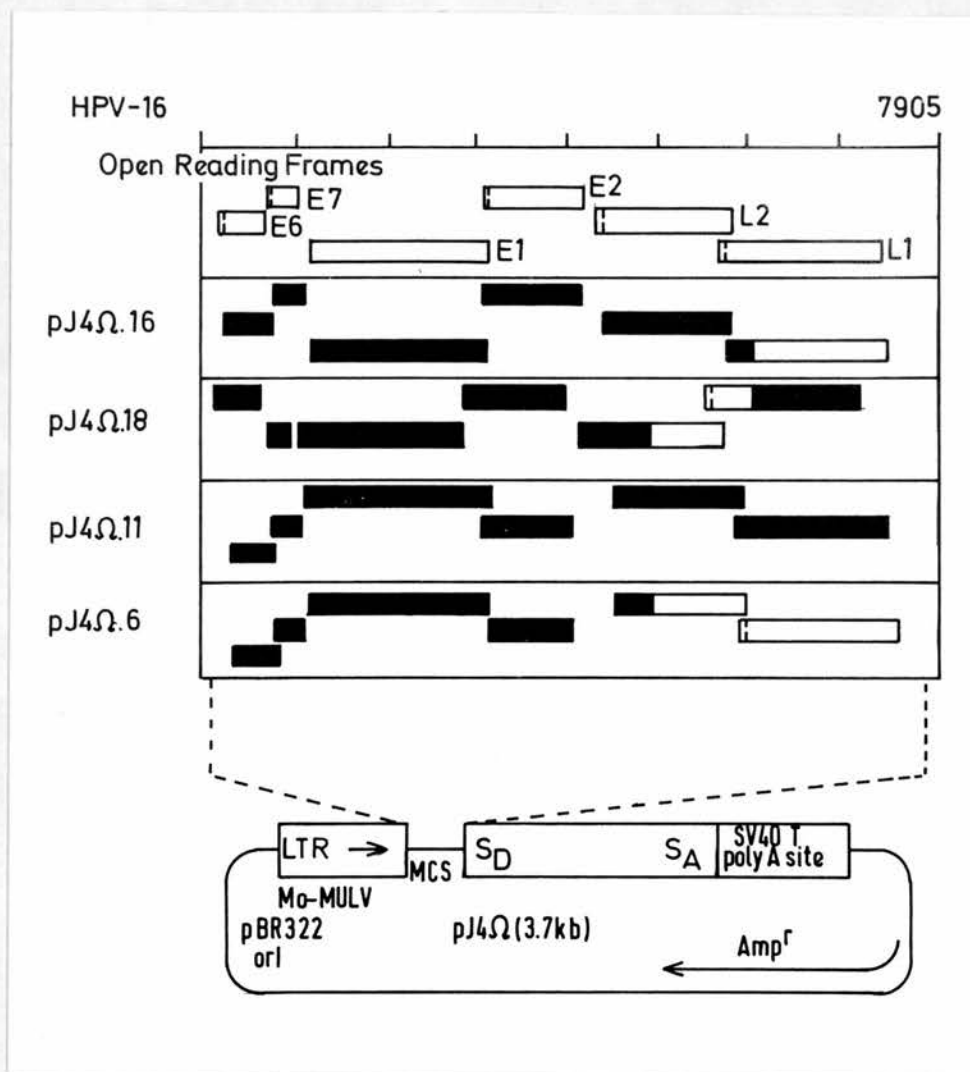


Figure 3.3

Plasmid maps showing construction of expression vectors containing HPV genomes. These were subcloned into the parent vector pJ40mega, at its multi cloning site (MCS), which is followed by a splice donor (SD) and splice acceptor (SA) site with a final SV40 T poly A site for termination of transcription. Upstream of the MCS there is a viral enhancer within the long terminal repeat (LTR) of the Moloney-Murine Leukaemia Virus (Mo-MULV). Four HPV types were used, 6b, 11, 18 and 16. Those regions of viral genomes used are indicated by shading of the relevant open reading frames, which correspond to the genes set out at the top of the diagram. All constructions contained most of the upstream regulatory region and the transforming genes E6 and E7 within a continuous DNA sequence.

### 3.3.2 CONSTRUCTION OF NEW PLASMIDS

Expression vectors for myc and ras oncogenes that contained a drug selection gene different from aph were synthesised for two purposes. First, independent clones of single oncogene transfectants derived by different transfection and selection protocols could be compared, so that similar phenotypic effects could be ascribed to the introduced gene rather than the experimental procedure. Second, serial transfection of myc and ras oncogenes into cells required separate selection methods. Thus, a DNA insert containing the hygromycin resistance gene (HmR) was introduced into the existing T24-ras expression plasmid pH05T1 to produce pHRH05T1, and also into the c-myc expression plasmid pMCGM1 to give pHRMCGM1.

#### 3.3.2.1 pHRH05T1 PLASMID

The pHRH05T1 plasmid (19.4 kb) was constructed by insertion of the Hind III linearised plasmid pHMR272 (Bernard et al, 1985) into the Hind III cleavage site in pH05T1 (figure 3.1). pH05T1 contains the mutated T24-ras oncogene linked to the SV40 promotor/enhancer element and the aph drug resistance gene, as well as the ampicillin resistance gene for bacterial selection (Spandidos and Wilkie, 1984). pHMR272 contains the Hmr gene (Kaster et al, 1977; Bernard et al, 1985) which confers resistance to hygromycin B, an aminoglycoside antibiotic which is

lethally toxic to mammalian and bacterial cells at appropriate doses. Thus, bacteria successfully transformed by recombinant plasmids were selected using agar with L-broth media containing both ampicillin and hygromycin B. Correct construction and orientation of the insert was confirmed by restriction mapping with EcoRI, BamHI, and HindIII which digested the plasmid into DNA fragments of the predicted lengths (figures 3.1 and 3.4).

#### 3.3.2.2 pHRMCGM1 PLASMID

pHRMCGM1 plasmid (19.7 kb) was constructed by insertion of the Hind III linearised plasmid pHMR272 into the Hind III cleavage site in plasmid pMCGM1, linking the hygromycin B resistance gene to the c-myc expressing sequences. Successfully transformed bacteria were selected using agar with L-broth media containing both ampicillin and hygromycin B. Correct construction and orientation of the insert was confirmed by restriction mapping with EcoRI, BamHI, and HindIII which digested the plasmid into DNA fragments of the predicted lengths (figures 3.2 and 3.5).

The plasmid structures are summarised in table 3.1.



Figure 3.4

Agarose gel electrophoresis showing restriction endonuclease mapping of the two newly constructed plasmids pHRH05T1 (two clones tested in tracks a and b, h and i) and pHRMCGM1 (two clones tested in tracks f and g, m and n). Enzymic digestions are also shown for the parent plasmids pH05T1 (tracks c and j), pHMR272 (tracks d and k) and pMCGM1 (tracks e and l). The first 7 tracks (a-g) show restriction with HindIII. The second 7 tracks (h-n) show restriction with BglII. The central tracks (M) are marker tracks consisting of lambda DNA digested by HindIII, giving bands of 23, 9.4, 6.6, 4.3, 2.3 and 2.0 kb. (A). HindIII cleaves pHRH05T1 into two fragments of 15.3 and 4.1 kilobases (a and b), pH05T1 into a single fragment of 15.3 kb (c), pHMR272 into a single fragment of 4.1 kb (d), pMCGM1 into a single fragment of 15.6 (e), and pHRMCGM1 into two fragments of 15.6 and 4.1 kb (f and g). BglII cleaves pHRH05T1 into three fragments of approximately 8, 7 and 4 kb (h and i), pH05T1 into two fragments of approximately 8 and 7 kb (j), pHMR272 into a single fragment of 4.1 kb (k), pMCGM1 into a single fragment of 15.6 kb (l), and pHRMCGM1 into two fragments of approximately 13 and 6.7 kb (m and n). These patterns of restriction enzyme digestion were used to confirm successful ligation and to determine orientation of the insert as shown in the plasmid maps.

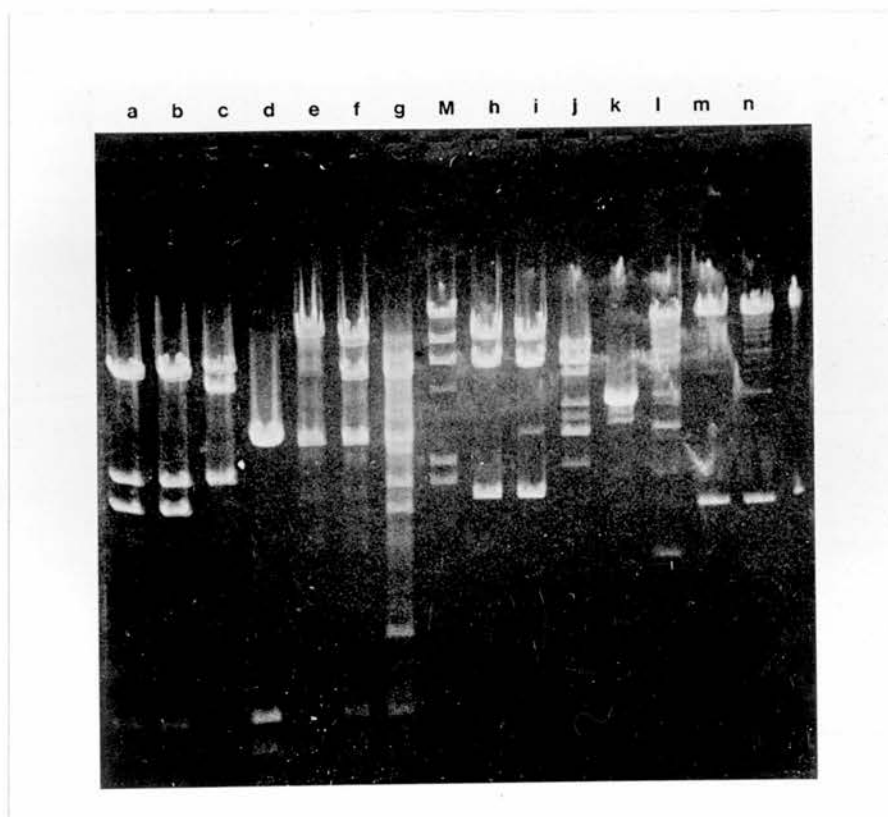


Figure 3.5

Agarose gel electrophoresis showing restriction endonuclease mapping of the two newly constructed plasmids pHRH05T1 (two clones tested in tracks a and b, h and i) and pHRMCGM1 (two clones tested in tracks f and g, m and n). Enzymic digestions are also shown for the parent plasmids pHO5T1 (tracks c and j), pHRM272 (tracks d and k) and pMCGM1 (tracks e and l). The first 7 tracks (a-g) show restriction with EcoRI. The second 7 tracks (h-n) show restriction with BamHI. EcoRI cleaves pHRH05T1 into six DNA fragments of approximately 7.4, 7.3, 2.2, 1.7, 0.5, and 0.3 kb (a and b), pHO5T1 into three fragments of 7.3, 5.8 and 2.2 kb (c), pHRM272 into 3 fragments of 3.3, 0.5 and 0.3 kb (d), pMCGM1 into two fragments of 12.7 and 2.9 kb (e), and pHRMCGM1 into five fragments of approximately 10, 6, 2.9, 0.5 and 0.3 kb (f and g). BamHI cleaves pHRH05T1 into three fragments of 11.2, 6.6, and 1.6 kb (h and i), pHO5T1 into two fragments of 8.7 and 6.6 kb (j), pHRM272 into one fragment of 3.9 kb and a small fragment lost from the gel (k), pMCGM1 into one fragment of 15.6 (l), and pHRMCGM1 into two fragment of approximately 18 and 1.5 kb (m and n). There is evidence of mild activity of BamHI at its known secondary recognition site (associated with high enzyme concentration) in tracks j, k, l, m and n. These patterns of restriction enzyme digestion were used to confirm successful ligation and to determine orientation of the insert as shown in the plasmid maps.

**TABLE 3.1****SUMMARY OF PLASMID SEQUENCES**

DESIGNATION	ONCOGENE	ENHANCER	DRUG RESISTANCE
pHO5T1	T24-ras	SV40	G418
pHRHO5T1	T24-ras	SV40	G418, HmB
pMCGM1	c-myc	Mo-LTR	G418
pHRMCGM1	c-myc	Mo-LTR	G418, HmB

Note: Transfected oncogenes included the T24-ras oncogene, a genomic human sequence of c-Ha-ras, mutationally activated at codon 12 (glycine to valine), and the c-myc, a genomic human proto-oncogene. The enhancers were derived from the viral genomes of Simian Virus 40 (SV40) and Moloney Murine Leukaemia virus long terminal repeat (Mo-LTR), and both were known to possess powerful promoter/enhancer activity. The drug selection genes conferred resistance to geneticin (G418) and Hygromycin B (HmB).



### 3.4 CELL LINE CONSTRUCTION AND CHARACTERISATION

#### 3.4.1 TRANSFECTION WITH ONCOGENE AND HPV DNA

The transforming potentials of high and low risk HPVs - alone or in combination with activated c-Ha-ras1 - were compared in cultured fibroblasts.

208F fibroblasts were electroporated with pJ40 expression plasmid vectors containing the cloned HPV DNA of types 6b, 11, 16 and 18. HPV genes were linked to strong heterologous promotor and enhancer sequences (Mo-MuLV LTR) in the pJ40 plasmid. The plasmid pHO5T1 contained T24-ras linked to the SV40 enhancer region and an aph gene. The plasmid pSV2NEO, containing only the aminoglycoside phosphohydrolase gene (aph), was cotransfected in a 1:10 ratio with HPV expression plasmids to confer resistance to geneticin (G418), for selection of positive transfectants. Combinations of HPV and ras expression plasmids in a 1:1 ratio were used. Numbers of surviving colonies after G418 selection were counted in three transfection experiments, and these colonies also demonstrated morphological changes (figure 3.6). Single colonies were subsequently isolated and established as clonal cell lines for further studies of tumour growth.



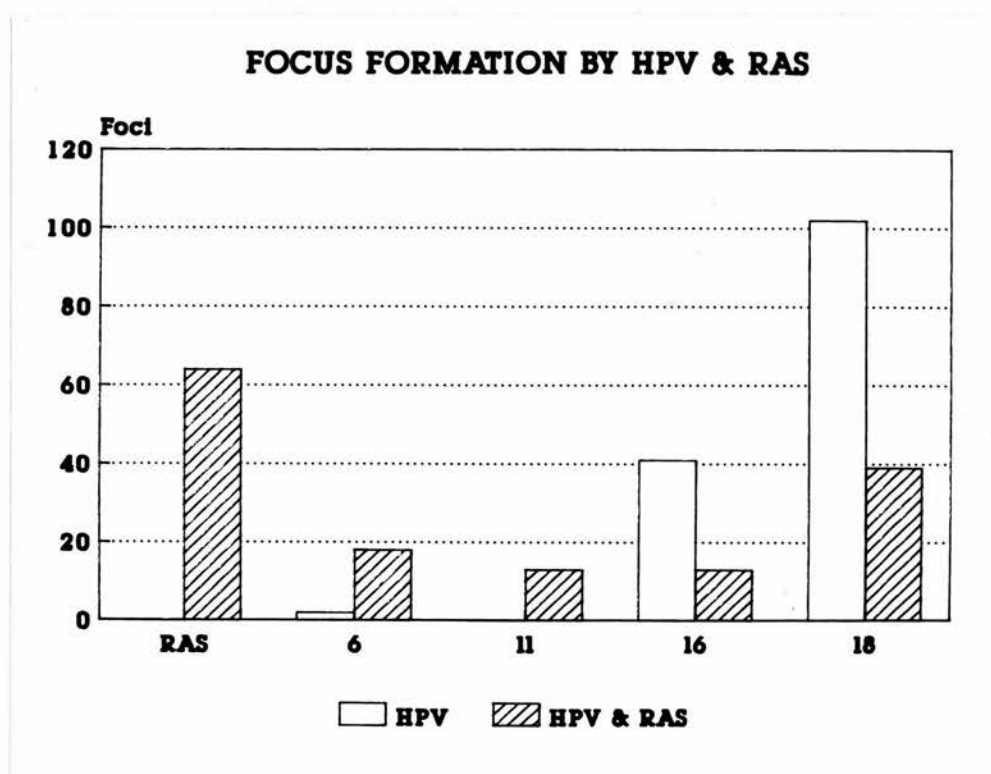


Figure 3.6

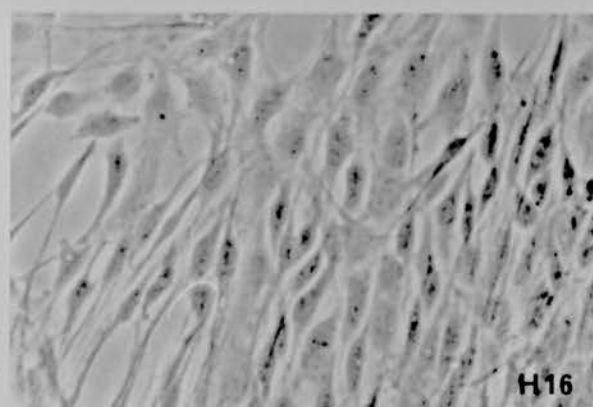
Bar chart of the number of morphologically transformed foci of cells following transfection of 208F cells with expression vectors (pJ40mega) for HPV genomes 6b, 11, 16 and 18, either alone or in combination with ras (pH05T1). Transfection of the ras expression vector alone is also shown for comparison.

Large numbers of foci were observed for HPV types 16 and 18, both alone and with T24-ras, and these showed morphological transformation from the parental flat, topo-inhibited monolayer cells to spindle-shaped, refractile cells which pile up (figure 3.7). No foci were seen with HPV 11 alone and only two non-transformed colonies with HPV 6b alone. The combinations of HPV types with T24-ras produced similar or lesser numbers of foci than T24-ras alone (figure 3.6).

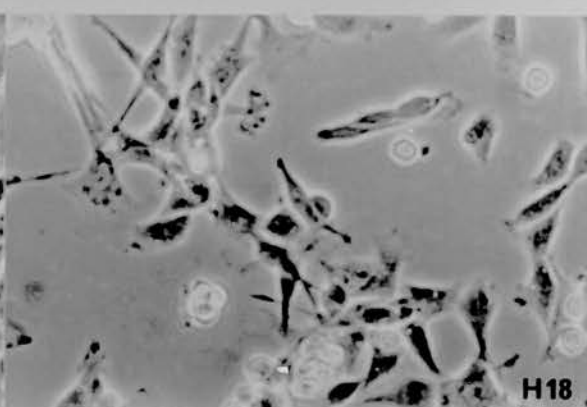
Fourteen cell lines were selected for study, all derivatives of 208F containing HPV, c-myc or T24-ras, alone or in certain combinations with each other (table 3.2). As a control, primary fibroblasts were prepared from freshly dissected rat lungs, and grown in vitro.

Figure 3.7

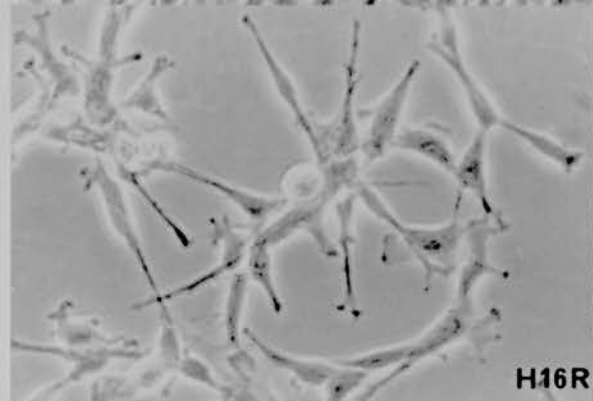
Morphological appearances of HPV transfected cell lines using phase contrast photo-microscopy. The four cell lines all appear morphologically transformed, H16R and H18R more so than H16 and H18, with spindle shaped, highly refractile cells that have overlapping cytoplasmic processes.



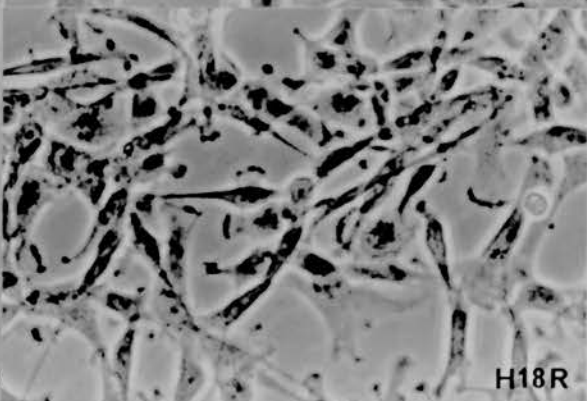
H16



H18



H16R



H18R

**TABLE 3.2 SUMMARY OF DNA SEQUENCES TRANSFECTED INTO CELL LINES**

CELL LINE	PARENT CELL	PLASMID	ONCOGENE OR HPV	ENHANCER	ISOLATION METHOD
208F	-	-	-	-	rat fibroblast line
T1 T2 T3	208F 208F 208F	pHO5T1 pHRHO5T1 pHRHO5T1	T24-ras T24-ras T24-ras	SV40 SV40 SV40	Ca/G418/Pop EP/Hmb/Clone EP/Hmb/Clone
M1 M7 M8	208F 208F 208F	pMCGM1 pHRMCGM1 pHRMCGM1	C-myc C-myc C-myc	MOLT MOLT MOLT	Ca/G418/Pop EP/Hmb/Clone EP/Hmb/Clone
MT4 MT7 MT9 MT10	M1 T1 208F 208F	pHRHO5T1 pHRMCGM1 pHRMCGM1 + pHRHO5T1 pHRMCGM1 + pHRHO5T1	C-myc + T24-ras T24-ras + C-myc C-myc + T24-ras C-myc + T24-ras	MOLT/SV40 SV40/MOLT MOLT/SV40 MOLT/SV40	EP/Hmb/Clone EP/Hmb/Clone EP/Hmb/Clone EP/Hmb/Clone
16 16R 18 18R	208F 208F 208F 208F	pJ40HPV16 + pSV2NEO pJ40HPV16 + pHO5T1 pJ40HPV18 + pSV2NEO pJ40HPV18 + pHO5T1	HPV 16 HPV 16 + T24-ras HPV 18 HPV 18 + T24-ras	MOLT MOLT/SV40 MOLT MOLT/SV40	EP/G418/Clone EP/G418/Clone EP/G418/Clone EP/G418/Clone

Note: Ca/G418/Pop: calcium phosphate transfection, selection with geneticin, and isolation as a mixed population of many colonies. EP/Hmb/Clone: transfection by electroporation, selection with hygromycin B, and isolation as single colonies expanded into clones. SV40 = Simian Virus 40 Enhancer. MOLT = Moloney Murine Leukaemia Virus Long Terminal Repeat Enhancer.

The parent cell line was 208F, an established rat fibroblast line with a hyperdiploid karyotype. This cell line was derived from the Fischer rat fibroblast line F2408 as a variant which was resistant to thioguanine after mutagenesis with ethyl-methane-sulphonate (Quade et al, 1979).

RFMCGM1 (called M1 hereafter) and RFH05T1 (called T1) were originally derived from 208F by Spandidos and Wilkie (1984). M1 contains the c-myc expression plasmid pMCGM1, and T1 contains the T24-ras expression plasmid pH05T1, both as a result of transfection with calcium phosphate and selection by geneticin. Multiple colonies of G418 resistant cells were cultured as mixed populations from which the cell lines were derived.

M7 and M8 were independently derived from 208F by transfection with the c-myc expression plasmid pHRMCGM1 using electroporation. Transfectants were selected with hygromycin B (to which the plasmid confers resistance). Unlike M1, they were clonally selected. T2 and T3 were similarly clonally selected independent 208F transfectants, obtained by electroporation with the T24-ras expression plasmid pHRH05T1, and selection by hygromycin B.

Four mixed T24-ras and c-myc transfectants were constructed. MT4 was derived from M1 cells by electroporation with the T24-ras expression plasmid

pHRH05T1. MT7 cell line was derived from T1 cells by electroporation with the c-myc expression plasmid pHRMCGM1. MT9 and MT10 were both derived from 208F by simultaneous electroporation with the c-myc and T24-ras expression plasmids (pHRMCGM1 and pHRH05T1). In these experiments transfectants were selected with hygromycin B and single colonies isolated as clones.

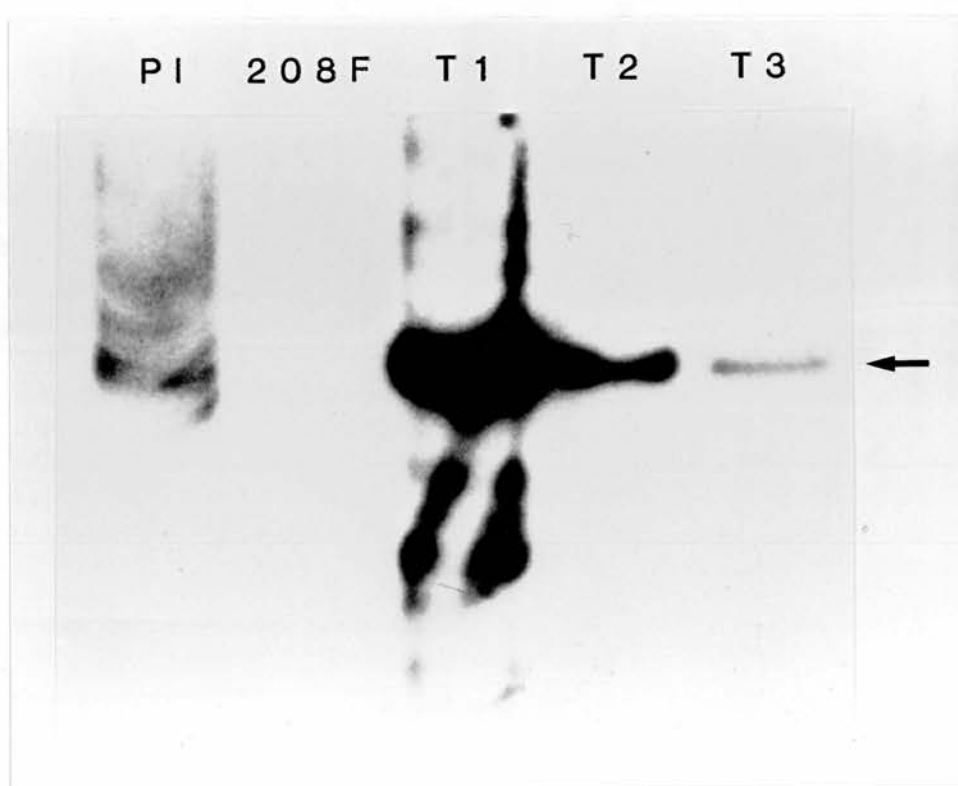
Four cell lines containing high risk HPV genomes, either alone or in combination with activated ras, were derived from 208F. H16 was constructed by cotransfection with the HPV 16 expression plasmid pJ40.16 and the drug resistance selection plasmid pSV2NEO, in a 10:1 molar ratio. Similarly, H18 was derived by cotransfection with the HPV 18 expression plasmid pJ40.18 and the drug resistance selection plasmid pSV2NEO, in a 10:1 molar ratio. H16R was constructed by cotransfection with the HPV 16 expression plasmid pJ40.16 and the T24-ras expression plasmid pH05T1 (which also contains the aph gene conferring geneticin resistance), in a 1:1 molar ratio. H18R cell line was similarly derived by cotransfection with the HPV 18 expression plasmid pJ40.18 and the T24-ras expression plasmid pH05T1, in a 1:1 molar ratio. Transfectants were selected with geneticin and single colonies isolated as clones.

#### 3.4.2 IDENTIFICATION OF TRANSFECTED DNA

Transfectants were assayed for exogenous DNA either by Southern blotting or PCR. In the 10 oncogene transfectants, c-myc and T24-ras sequences were detected using Southern blots prepared from cell line DNA digested by HindIII, and probed with HindIII-restricted plasmids pHRH05T1 (T1, T2, T3, MT4, MT7, MT9 and MT10), or pHRMCGM1 (M1, M7 and M8). The 10 oncogene-transfected cell lines exhibited bands of the appropriate sizes expected of the introduced oncogene fragments and these were not seen in 208F (figure 3.8). Bands of other sizes and faint bands present in 208F may have represented plasmid DNA sequences disrupted during integration, or endogenous ras or myc DNA sequences. In the four HPV transfectants, transfected DNA was detected by PCR assay, either using the specific primers for HPV 16 or 18 described in chapter 2, or using human specific Ha-ras primers described by Bos et al, (1987) (figure 3.9).



A



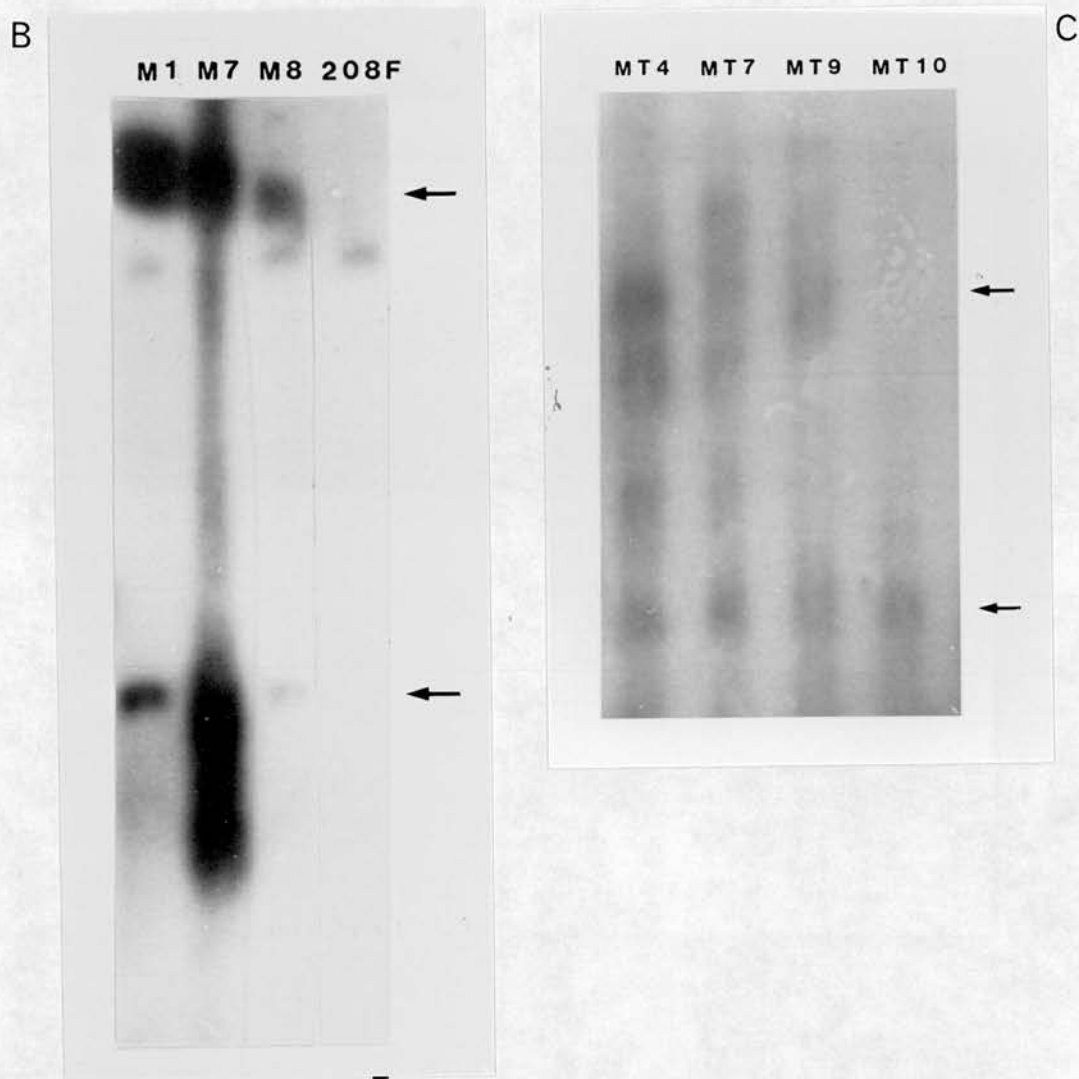


Figure 3.8

Southern blots of oncogene transfectants. (A) The three ras transfectants T1, T2 and T3 are compared with the parent cell line 208F and a plasmid DNA control (pHRH05T1). The blot was probed using labelled pHRH05T1 plasmid DNA. The parent cell line is negative but the three transfectants contain the ras sequence. The strongest hybridisation signal is seen in T1, followed by T2 and lastly T3 with the weakest signal. (B) The three c-myc transfectants M1, M7 and M8 are compared with the parent 208F. There are two bands present in the transfectants which are absent in the parent (arrows) which demonstrate transfection of the c-myc oncogene. Labelled plasmid pHRMCGM1 was used as the probe. (C) The four co-transfectants MT4, MT7, MT9 and MT10 were probed with the ras probe using labelled pHRH05T1. This shows the presence of bands at 2 dominant position (arrows).

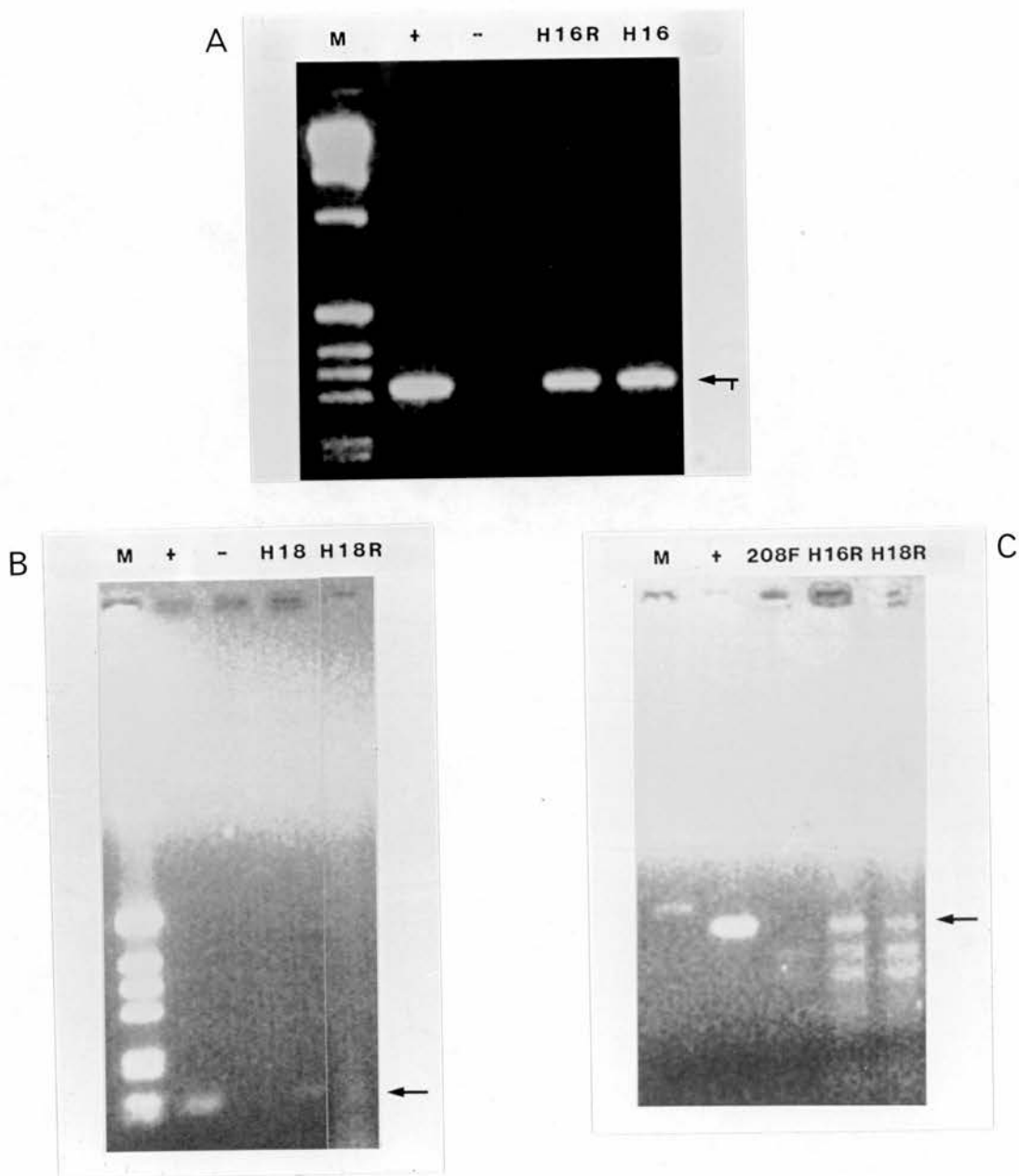


Figure 3.9

Agarose gel electrophoresis showing detection of transfected DNA using PCR. (A) HPV 16 primers were used to generate an amplified product of 316 base pairs in the cell lines H16R and H16. A 1 kb ladder marker track (M), positive control (+) of cloned HPV16 DNA and a negative template free control (-) are included. (B) HPV 18 primers were used to generate an amplified product of 144 base pairs in the two cell lines H18 and H18R. Similar positive and negative controls are included. (C) The set of *ras* primers developed by Bos et al (1987) were used to generate amplified products of 60 base pairs from the human c-Ha-*ras*1 gene in the cell lines H16R and H18R, which were not present in the parent 208F. A positive control (+) pH05T1 plasmid template is included.

### 3.4.3 ONCOGENE EXPRESSION

A new assay based on reverse transcription and polymerase chain reaction (rT-PCR) was developed for the detection of c-myc and c-Ha-ras1 mRNA, using a similar approach to that described in chapter 2 for primer selection. Species specific primers were chosen in exons 2 and 3 of human c-myc (Watson et al, 1983; Battey et al, 1983), and exons 3 and 4 of human c-Ha-ras (Santos et al, 1982; Reddy et al, 1982; Capon et al, 1983). As an internal reference, primers in exons 3 and 4 of beta actin were also used (Nudel et al, 1983; Ng et al, 1985). These "exon-connecting" primers allowed clear distinction between products amplified from cDNA (introns spliced out confirming transcription) and genomic DNA (introns retained). The c-myc primers amplify a sequence of 1610 bp in genomic DNA and 234 bp in cDNA reverse transcribed from RNA. The primers for c-Ha-ras1 amplify a product of 800 bp from genomic DNA and 103 bp from cDNA. Homology to rat sequences was less than 80% for both oncogenes (tables 3.3 and 3.4).

The rT-PCR assay was applied to RNA extracted from single oncogene transfectants after exclusion of significant RNA degradation by formaldehyde-agarose gel electrophoresis. 208F was negative for both myc and ras expression. Bands of the appropriate sizes for myc transcripts were generated from M7 and M8 RNA samples, and similarly for ras mRNA from T1, T2 and T3, and for control actin

transcripts from the 4 newly constructed single oncogene transfectants M7, M8, T2 and T3 (figure 3.10).

TABLE 3.3

OLIGONUCLEOTIDES

GENE	EXON	SPECIES <sup>b</sup>	PRIMER	SEQUENCE (5'→3')	%GC	HOM <sup>c</sup>	IBP <sup>d</sup>	POSITION
c-myc	2	H	primer 1	GCG CCT CGC AAG ACT CCA GC	70	60	2	5143-5162
c-myc	3	H	primer 2	CTC CAG CAG AAG GTG ATC CAG	57	52	4	6732-6752
c-Ha-ras1	3	H	primer 1	TCA GGA CCT CGC CCG AAG CTA C	64	77	4	2473-2494
c-Ha-ras1	4	H	primer 2	CCG GAT CTC ACG CAC CAA CG	65	75	4	3253-3272
$\beta$ -actin	3	H/R	primer 1	TAC AAT GAG CTG CGT GTG GC	55	100		1601-1620(R) 1495-1514(H)
$\beta$ -actin	4	H/R	primer 2	CAT GGC TGG GGT GTT GAA GG	60	100	2	2171-2190(R) 2042-2061(H)

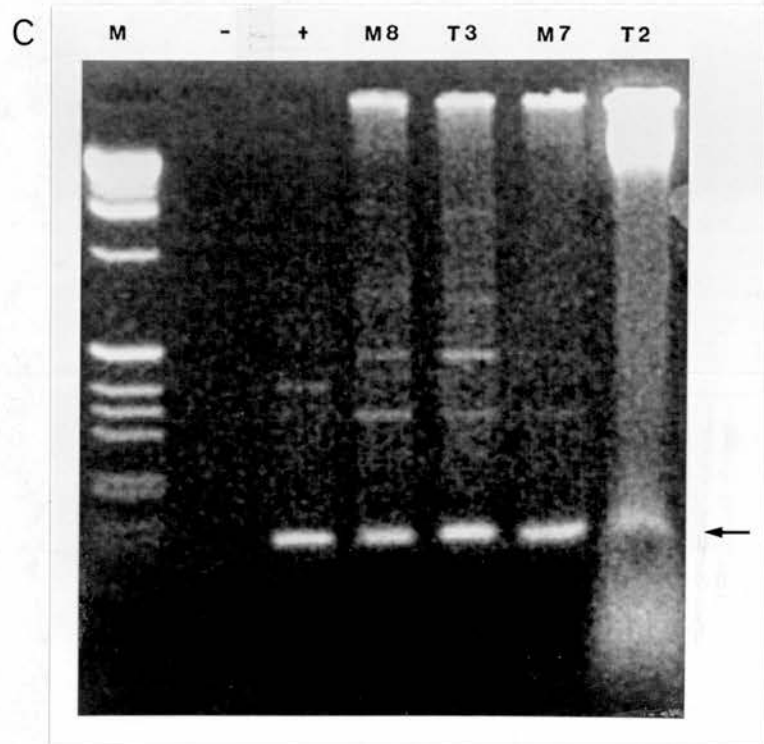
NOTES: EMBL nomenclature used throughout. (a) NC = non-coding, (b) species = human (H) or rat (R), (c) HOM = homology with comparator, (d) IBP = internal base pairings of predicted oligonucleotide secondary structure.

**TABLE 3.4**

**AMPLIFIED PRODUCTS**

<b>GENE</b>	<b>EXONS</b>	<b>SPECIES</b>	<b>DNA/RNA</b>	<b>SEQUENCES AMPLIFIED</b>	<b>PRODUCT LENGTH</b>	<b>DNA/RNA PRODUCT RATIO</b>
c-myc	2-3	H	GENOMIC	5143-6752	1610	6.88
c-myc	2-3	H	mRNA	5143-5277/6654-6752	234	
c-Ha-ras1	3-4	H	GENOMIC	2473-3272	800	7.77
c-Ha-ras1	3-4	H	mRNA	2473-2533/3231-3272	103	
$\beta$ -actin	3-4	H	GENOMIC	1495-2061	567	4.50
$\beta$ -actin	3-4	H	mRNA	1495-1587/2029-2061	126	
$\beta$ -actin	3-4	R	GENOMIC	1601-2190	590	
$\beta$ -actin	3-4	R	mRNA	1601-1693/2158-2190	126	4.68

NOTES: EMBL nomenclature used throughout.





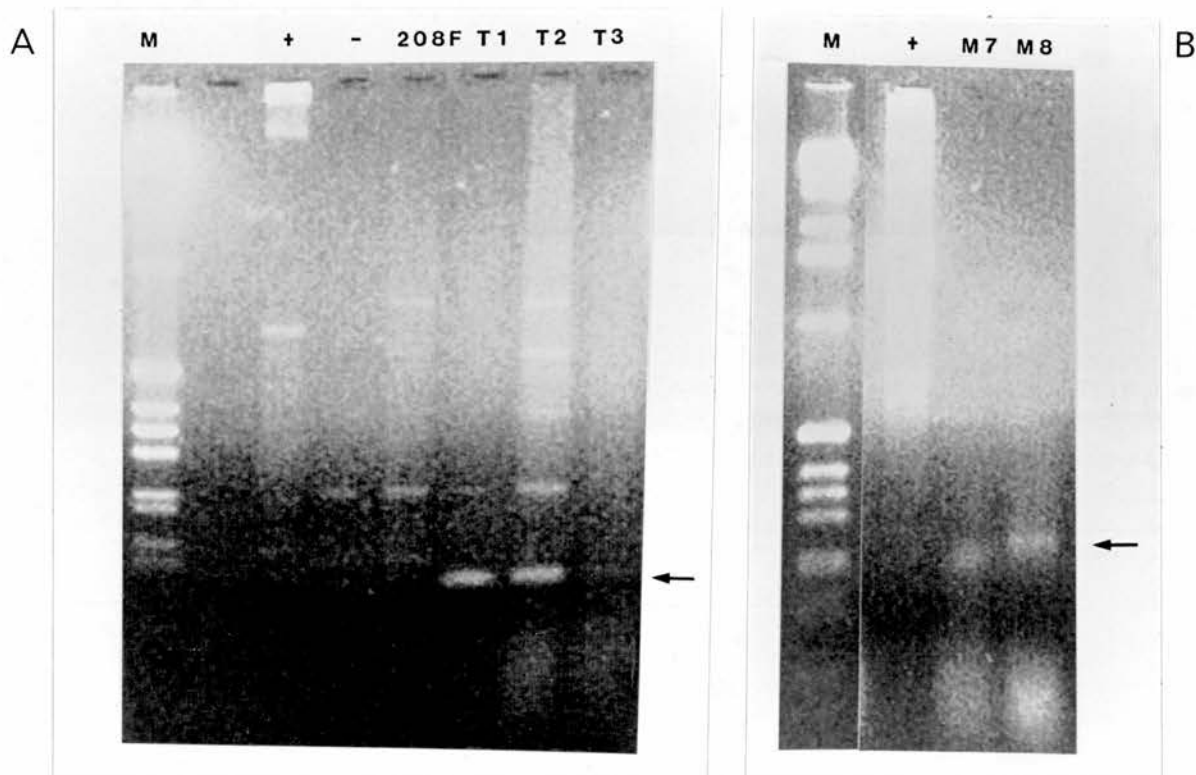


Figure 3.10

Agarose gel electrophoresis of products generated by the reverse transcription - polymerase chain reaction assay used to detect specific RNA species within cells. (A) ras specific RNA is detected in the three ras transfectants T1, T2 and T3. This amplified product measures 103 bp in length (arrow). A negative template free control (-), a positive control (+) using as template the genomic ras gene in plasmid pH05T1 (which generates a DNA amplified product of 800 bp), and a 1 kb ladder of marker track are included. (B) myc primers were used to generate reverse transcribed and PCR amplified products for M7 and M8, of length 234 bp (arrow). The positive control (+) used as template the genomic c-myc sequence in plasmid pMCGM1 (which generates a DNA fragment of 1610 bp). A 1 kb ladder marker track is included. (C) Detection of expression of the actin gene was used as a control. Primers specific for actin RNA sequences were used to generate reverse transcribed and PCR amplified products of length 126 bp (arrow) for the cell lines M8, T3, M7 and T2. A positive control (+) was included which used as template the actin cDNA cloned in a plasmid. A template-free negative control (-) and a 1 kb marker track are also included.

Validation of this new assay and independent confirmation of c-myc expression in the 2 newly constructed myc transfectants was obtained using dot blot hybridisation of extracted RNA. M7 and M8 gave strongly positive hybridisation signals with a myc labelled probe (8.4 kb DNA fragment from EcoRI - HindIII digested pMC41C1 plasmid, containing the 3 myc exons), whereas the parent (208F) gave a weaker signal, as expected of an actively proliferating cell line not transfected with myc. Approximately equal loading of cellular RNA was confirmed by hybridisation with a ribosomal sequence probe (10 kb DNA fragment from HindIII digested pXLR101NH plasmid [Dr N Hunter, personal communication], containing *Xenopus* ribosomal sequences) (figure 3.11).

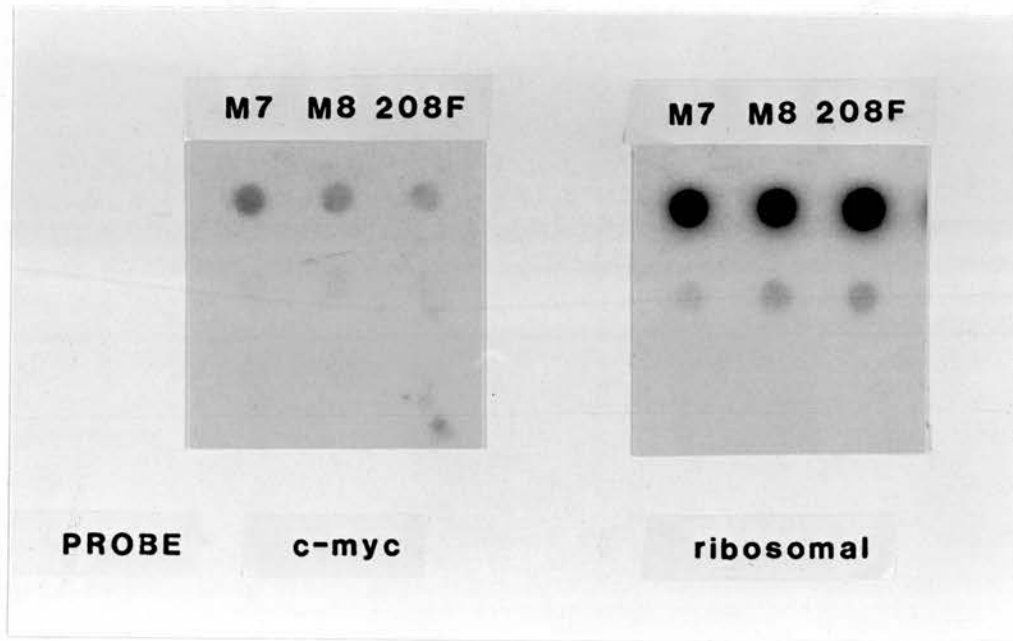


Figure 3.11

Dot blot hybridisation of RNA extracted from the M7 and M8 cell lines which contain the *c-myc* oncogene and also the parent 208F line. On the left the blot was probed with labelled *c-myc* DNA, and the hybridisation signals are stronger for M7 and M8 compared with 208F. On the right, a duplicate blot was hybridised with a labelled ribosomal DNA probe and this showed approximately equivalent loading of RNA from the three cell lines.

### 3.5 GROWTH PARAMETERS OF ONCOGENE TRANSFECTANTS

#### 3.5.1 GROWTH IN CULTURE

##### 3.5.1.1 MORPHOLOGICAL TRANSFORMATION PHENOTYPES

The morphological appearances of oncogene and HPV transfectants were recorded by phase contrast photomicroscopy (figures 3.12 and 3.7). Three criteria were used to assess morphological transformation: intercellular relationships including focus formation and overlapping cytoplasmic processes, cell shape, and refractile appearance. These features, although open to bias from subjective observation, may be important features of tumour cells, in that they may represent alterations in cytoskeletal architecture, contact-inhibition of growth and changes of surface attachments or interactions of tumour cells.

##### i. Parental Established Rat Fibroblast (208F).

The parental 208F cell line demonstrated topo-inhibition of growth in culture by forming a flat confluent monolayer with no gaps between adjacent cells. The cells did not grow in foci or overlap each other, and had a non-refractile polygonal appearance, typical of non-transformed cells.

Controls for subsequent experiments included untransfected 208F cells, cells electroporated with no

DNA, or transfected with the drug selection vector only (pSV2NE0), all of which showed no evidence of morphological transformation.

ii. C-myc transfectants (M1, M7, M8).

The three c-myc transfected cell lines demonstrated similar morphological features in vitro. The flat spindle-shaped cells were only mildly refractile, and were able to form near-confluent monolayers. At both high and low cell densities there was very tight side-to-side clustering of cells, producing a fascicular appearance. At low cell densities a limited degree of overlap of cellular cytoplasmic processes was also seen at the expanding edges of cell colonies, but foci of greater than 2-3 cells high were not observed.

iii. T24-ras transfectants (T1, T2, T3).

The three T24-ras transfected cell lines showed similar characteristic morphological features when growing in culture at low cell densities: tubular, spindle-shaped cells, showing marked refractility, and piling up in foci, with adjacent cells overlapping, often 2-4 cells high. At high cell densities, T1 and T2 tended to overlap to a greater degree, and did not form a confluent carpet of cells, whereas T3 formed a dense near-confluent carpet of moderately refractile cells with considerable cellular overlap within it.

iv. Serial myc-ras transfectant (MT4, MT7).

The MT4 cell line exhibited a similar appearance to its immediate parent, the c-myc expressing line M1, with mildly refractile cells growing in fascicular patterns to form a monolayer. The tendency for cells to overlap in foci at lower cell densities was not seen at higher cell densities.

The MT7 cells showed similar morphological appearance to its immediate parent, the T24-ras expressing line T1, at low densities, they grew in foci of overlapping cells, at high densities they formed a dense carpet with gaps between adjacent cells. The cells were spindle-shaped and highly refractile.

v. myc-ras cotransfectants (MT9, MT10).

MT9 and MT10 both formed confluent monolayers with no gaps between adjacent cells, but with a limited degree of piling up of cells at low cell densities; these cell lines tended to grow not in foci, but in a more diffuse manner. The cells were moderately refractile.

Figure 3.12

Appearances of morphologically transformed cells recorded by phase contrast photomicroscopy. (A) Three c-myc transfectants, M1, M7 and M8 show a mild degree of transformation with mild refractility but a more striking fascicle formation whereby cells line up in their longitudinal axes. The 3 ras transfectants T1, T2 and T3 show moderate to high degrees of morphological transformation, with refractility, spindle cell formation and overlapping of cytoplasmic processes. T1 and T2 show greater transformation than T3.

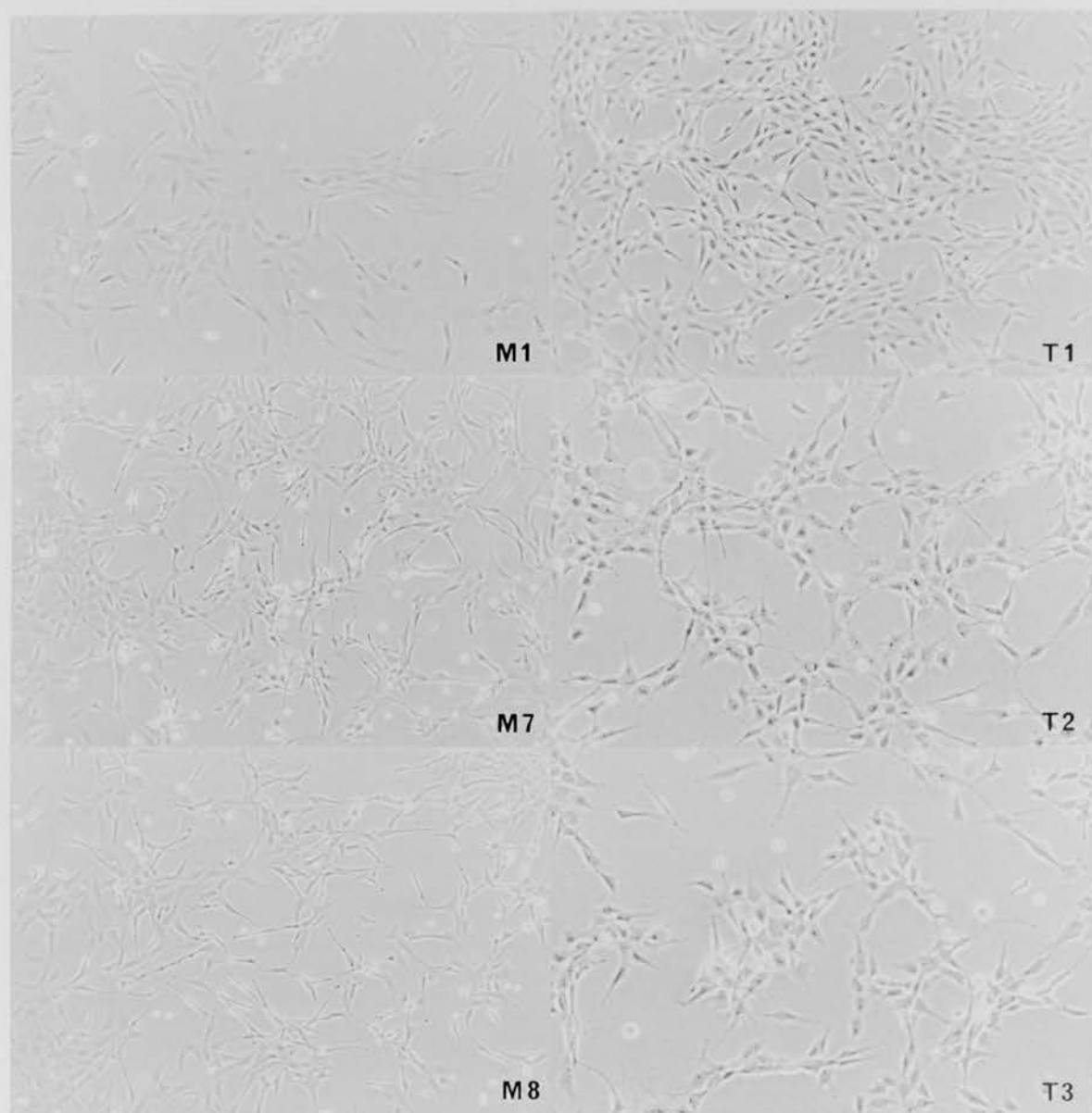
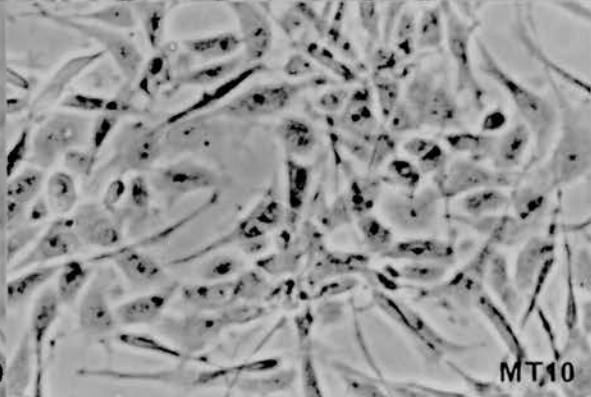
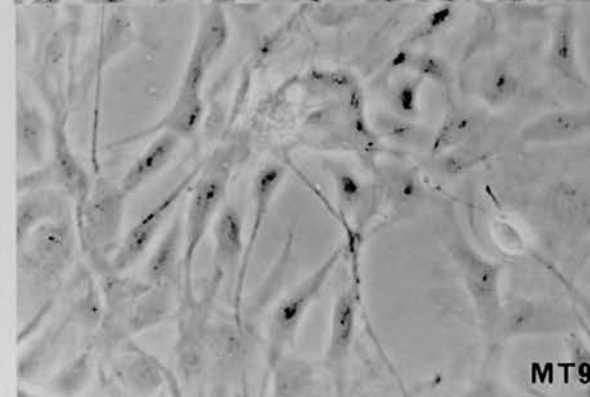
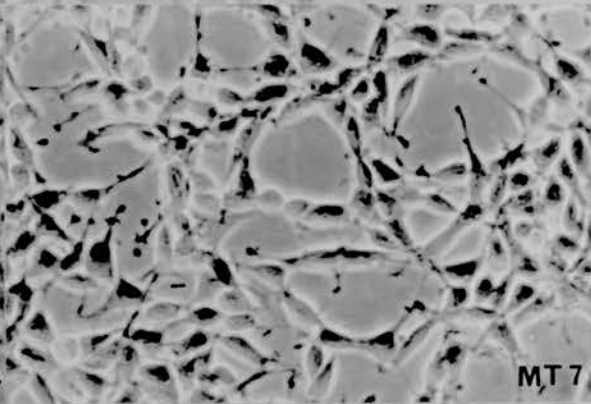
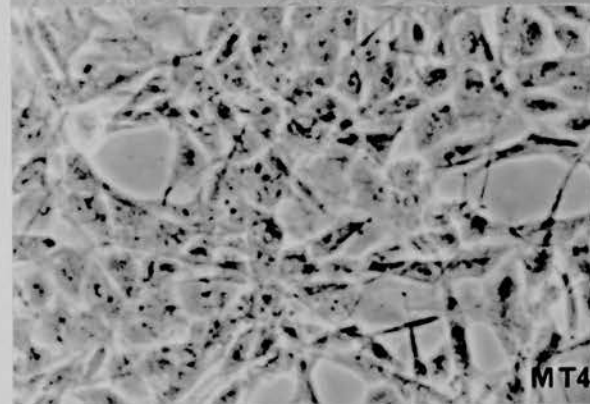
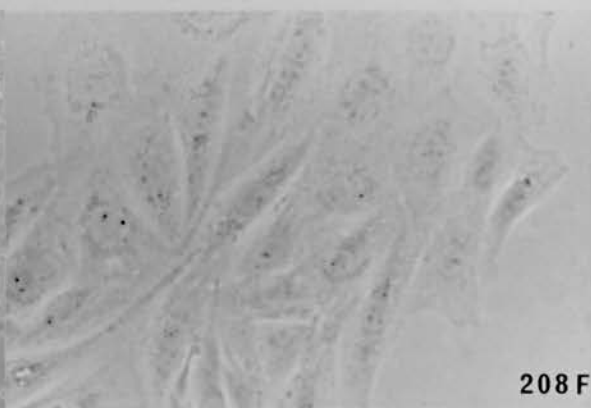
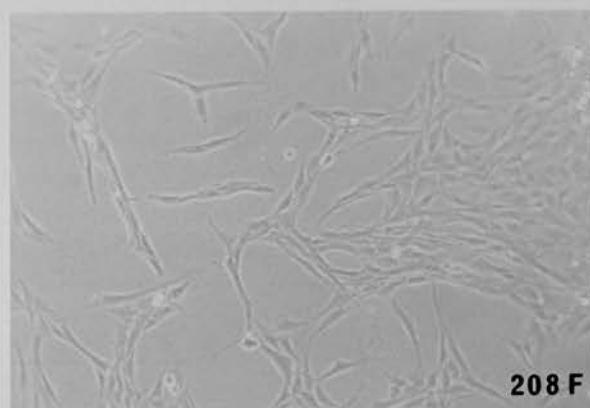




Figure 3.12

(B) The parent cell line, 208F, at medium and high power magnification is compared with the 4 double oncogene transfectants MT4, MT7, MT9 and MT10. Varying degrees of morphological transformation are seen. MT7 shows the greatest degree of transformation with MT9, MT10 and MT4 all showing similarly moderate degrees of morphological transformation.



vi. HPV 16 and 18 transfectants (H16 and H18).

Both high risk HPV transfectants formed confluent monolayers with no gaps between adjacent cells, and a limited degree of piling up of cells at low densities. Moderate refractility of the cells was observed, but fascicular formation was not seen.

vii. HPV 16 + ras / HPV 18 + ras transfectants (H16R and H18R).

The combined high risk HPV plus ras transfectants displayed a highly transformed morphology at both low and high cell densities, forming foci of overlapping cells. Individual cells were spindle-shaped and highly refractile. It was not possible to morphologically distinguish these from fibroblasts transfected with ras alone (T1 or T2).

viii. Primary Rat Lung Cells

The flat round or polygonal cells were non-refractile and formed a confluent monolayer, demonstrating topoinhibition. They showed no piling up, or overlapping of cells and no focus formation (data not shown).

Control experiments in which 208F cells either underwent electroporation without any DNA, or were transfected with a "dummy" plasmid (pBR322), showed no morphological transformation afterwards and died during aph or HmB drug selection. Other experimental studies have also included

controls in which 208F cells were transfected with similar plasmid sequences without an oncogene, but containing the same promoter/enhancer elements, drug selection genes and bacterial sequences. These experiments showed no morphological transformation (Spandidos and Wilkie, 1984; Spandidos, 1985).

#### 3.5.1.2 INVESTIGATION OF CELL DEATH IN CULTURE

##### 3.5.1.2.A MORPHOLOGICAL STUDY OF CELL DEATH

Ultrastructurally, cells recovered from the monolayers of all cell lines showed similar features (data not shown). Some lines (to be detailed later) also released cells into the medium in substantial numbers. These showed all the cardinal features of apoptosis (figure 3.13 parts I and II). Cytoplasmic changes included shrinkage in cell volume with compaction of morphologically normal organelles, formation of dilated endoplasmic reticulum vesicles some of which appeared to fuse with the cell membrane, and the appearance of semi-crystalline arrays of ribosomal particles. Nuclear changes included condensation of chromatin, with peripheral margination into crescentic caps or toroidal structures, nucleolar segregation and dispersion. Many cells showed fragmentation of the nuclear structure into discrete spherical masses of chromatin, numbering between three and twelve. Phagocytosis of apoptotic bodies by monolayer cells was only occasionally observed.

Figure 3.13

(I). Ultrastructural appearances of viable and apoptotic transfectants. (A) Two viable fibroblasts taken from the monolayer. The nuclei display both euchromatin and heterochromatin with one or more nucleoli. (B) In apoptosis, the chromatin condenses around the periphery of the nucleus forming either toroids or crescentic caps, and the nucleolus undergoes a characteristic pattern of disintegration. (C) The fibrillar centre of the nucleolus is conserved, following dissociation of the dense fibrillar and granular components seen in the central zone of the nucleoplasm. (D) The conserved fibrillar centre is seen adjacent to the condensed chromatin at the nuclear margin, separated by a thin layer of nucleoplasm which is less electron dense. In the cytoplasm there is vacuolation around the periphery of the cell just below the plasma membrane. Some of these vacuoles appear to fuse with the cell membrane. (E) Nucleolar disintegration into the three components is apparent. (F) In phase 2 of apoptosis, the nucleus splits up into smaller spheres of darkly staining condensed chromatin seen here scattered through the cytoplasm of the cell.

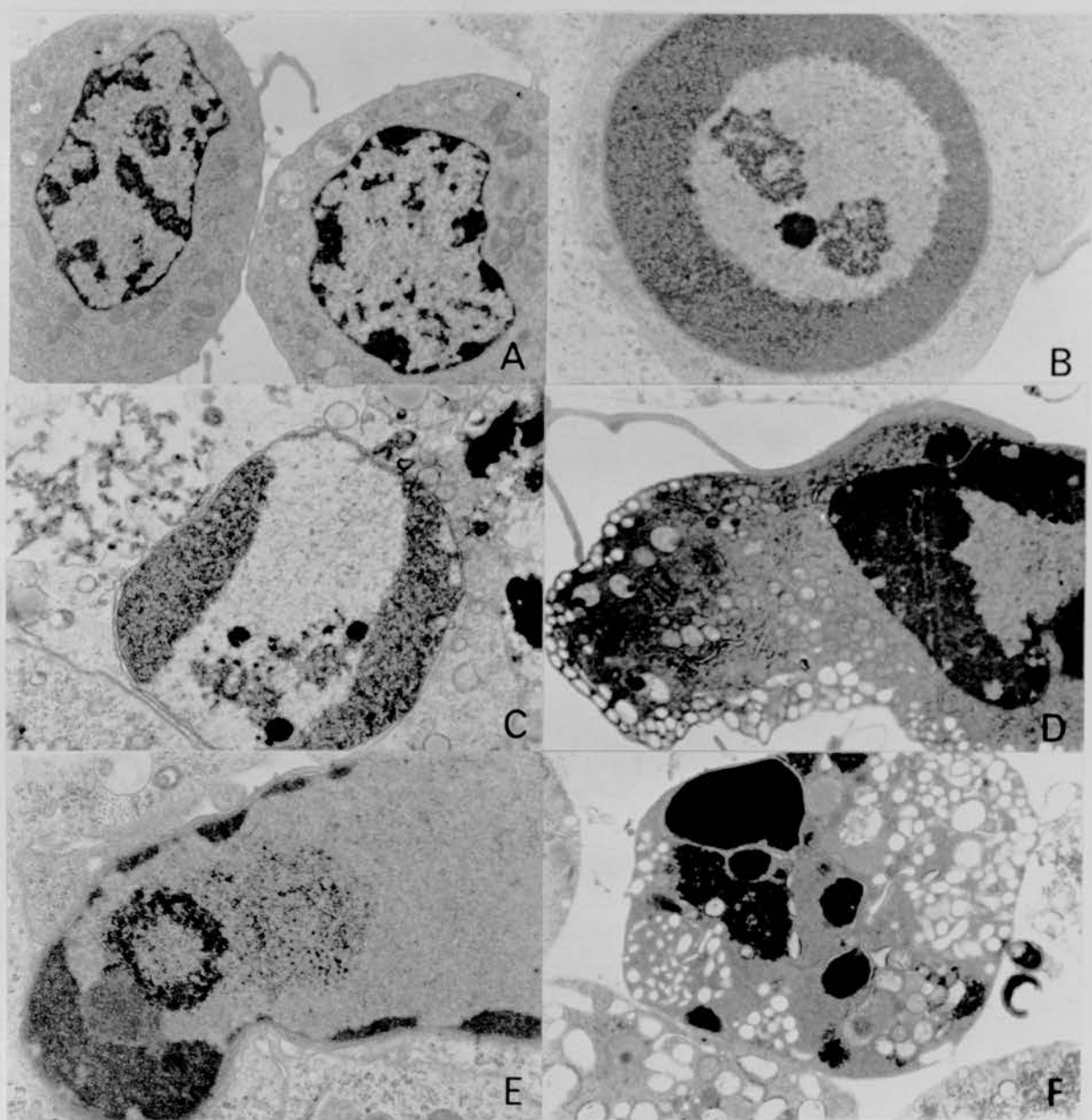
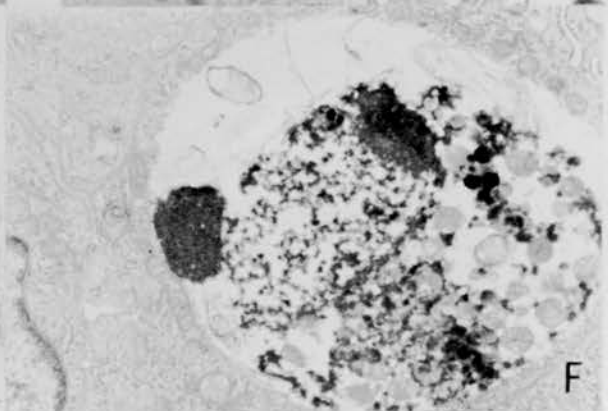
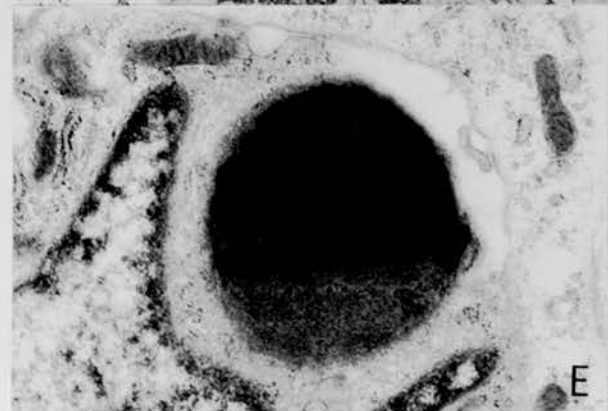
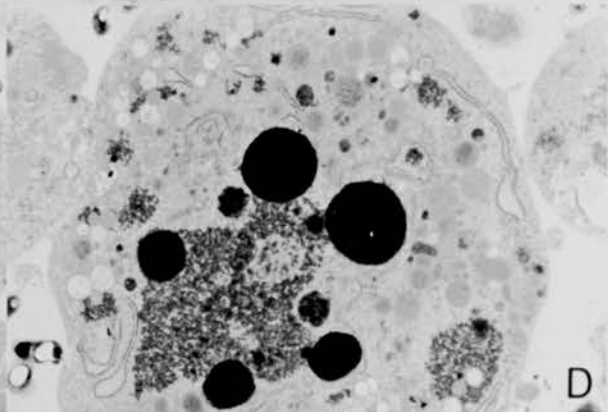
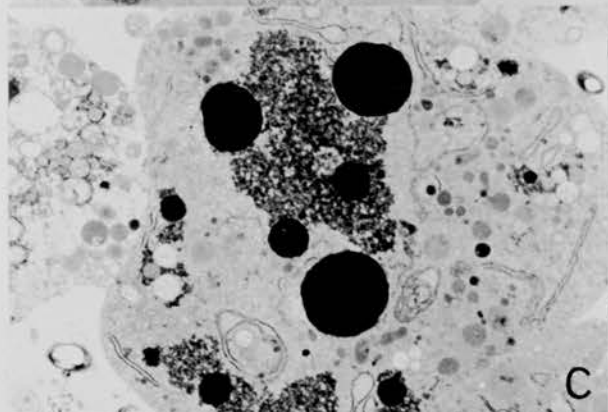
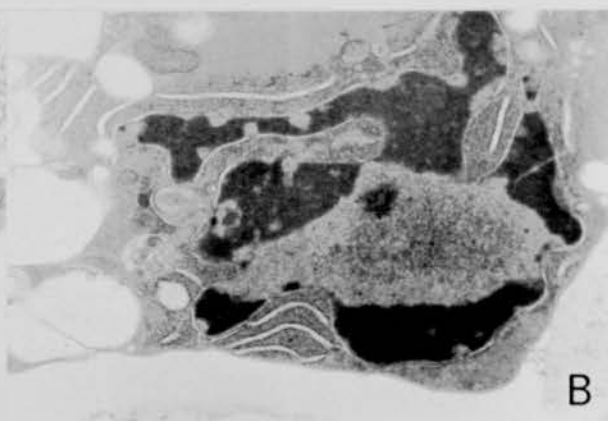
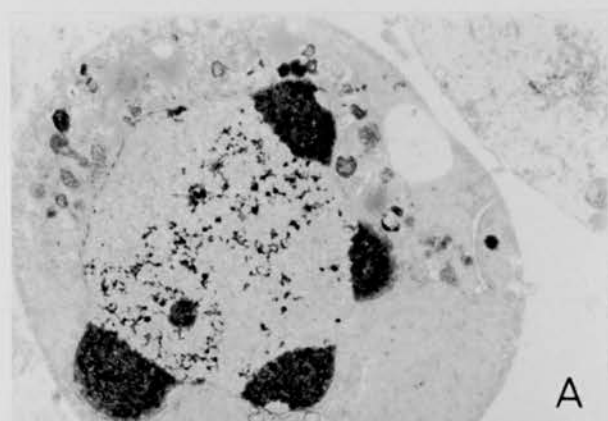


Figure 3.13

(II). (A) Prior to fragmentation of the apoptotic nucleus, the condensed chromatin often aggregates in dense clumps at the periphery of the nucleus. (B) The nucleus appears to develop pseudopodium-like structures filled with condensed chromatin as it splits up into separate spheres of chromatin. (C) Multiple densely staining spheres of chromatin are formed and ribosomes are often seen detached from the endoplasmic reticulum, aggregated into semi-crystalline arrays. (D) The apoptotic cell fragments into several small apoptotic bodies which may or may not contain condensed chromatin, and some of these are phagocytosed by adjacent cells in the monolayer (E), or may be released into the culture medium. (F) Phagocytosed apoptotic bodies eventually undergo degradation, and the residual bodies may resemble fragments of necrotic cells.







Most apoptotic cells were released into the culture medium and after an extended period these showed secondary degenerative changes, such as membrane rupture and swelling. In contrast, primary necrosis of cells was observed very seldom.

Fluorescence microscopy of wet preparations of cells released into the media, stained in acridine orange, showed closely similar nuclear morphology of apoptotic cells compared to electron microscopy. These were clearly demonstrated as DNA stained with acridine orange emitted yellow-green fluorescence. In particular, there was chromatin condensation into single nuclear structures or multiple spherical masses. In a small proportion of apoptotic cells the arrays of ribosomal particles, were discernable by acridine orange staining, as single-stranded RNA emitted red fluorescence (figure 3.14). This morphological correlation validated the technique of identification of large numbers of apoptotic cells by fluorescence microscopy, and their differentiation from viable cells. Thus, whereas electron microscopy was of use in defining qualitative aspects of apoptosis, fluorescence microscopy permitted quantitation.

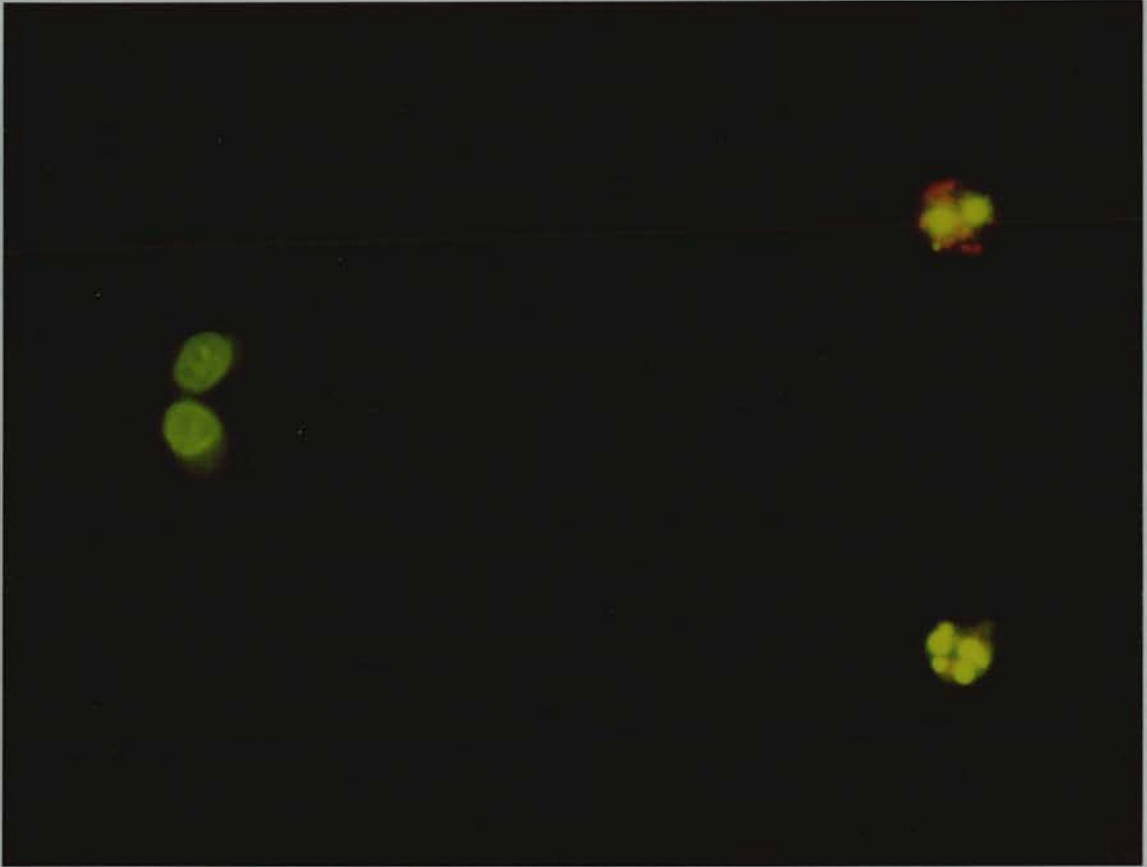


Figure 3.14

Comparison of viable (left) and apoptotic (right) cells released into the culture medium by a myc transfectant. The cells were stained with acridine orange and viewed under ultraviolet light using fluorescence microscopy. In viable cell nuclei, internal structure can be discerned, including denser staining of the heterochromatin around the periphery of the nucleus and brighter staining of the nucleolus. In the apoptotic cells, nuclei have often fragmented into multiple small spherical particles of condensed chromatin which stain a brighter yellow/green fluorescence. Ribosomal arrays in apoptotic cells may also stain as bright red dots (acridine orange stains single stranded nucleic acids red).

### 3.5.1.2.B ENDOGENOUS ENDONUCLEASE ACTIVITY IN APOPTOTIC AND VIABLE CELLS

To determine whether the structural form of DNA within the cellular bodies released into culture medium was that of apoptosis, cell bodies were harvested from the media overlying confluent cultures of cells. DNA was extracted and analysed by agarose gel electrophoresis. DNA from 2 samples of released cell bodies of the c-myc oncogene transfectant M1 is shown as an example (figure 3.15A). The typical "chromatin ladder" of DNA bands representing oligonucleosomal fragments was seen, indicating activation of the endogenous endonuclease associated with apoptosis and further confirming that these were apoptotic cells.

Expression of this endonuclease activity by viable cells, taken from the monolayer, was examined for the single myc and ras oncogene transfectants. Glycerol purified nuclei were prepared from monolayer cells grown at high or low serum concentrations for 4 hours prior to analysis. These were tested in an autodigestion assay, designed to allow digestion of chromatin within the nuclei by the endonuclease, if present, following its activation by manipulation of the ionic environment. The nuclease activity of the endogenous endonuclease associated with apoptosis is known to be sensitive to the 2 divalent cations calcium and magnesium, and these must both be present at critical concentrations for full

enzymic activity (nuclease activity can also be inhibited by zinc ions).

DNA was extracted from autodigested nuclei and analysed by agarose gel electrophoresis. Using nuclei derived from cells grown in high serum concentrations (10% HINCS) and autodigested in the presence of both cations this assay showed chromatin cleavage into oligonucleosomal fragments in M1, M7 and M8, but not in 208F, or T1 (figure 3.15B). The oligonucleosomal sized DNA bands from myc transfectants were sometimes seen on a background of DNA smears. Only high molecular weight DNA, with minimal or no low molecular weight DNA smears or oligonucleosomal fragments, were seen in the samples of 208F and T1. This suggested that M1, M7 and M8 viable cells expressed endonuclease, whereas T1 and 208F viable cells did not. There was markedly less nuclease activity in the myc transfectant nuclei if only one cation (calcium) was present, and in the case of M8, a cell line with high endonuclease activity, there was complete inhibition of nuclease activity by zinc ions.

Low endonuclease activity was present in T2 and T3 nuclei prepared from cells grown in high serum (10% HINCS) concentrations, but this activity was markedly increased by growth in low serum (0.05%) for 4 hours prior to analysis (figure 3.15C). At high serum there was mild cleavage of chromatin into mostly large oligonucleosomal fragments, but at low serum a greater proportion of

digested chromatin appeared as small oligonucleosomes, in particular the bottom two rungs of the chromatin ladder, representing mono- and di-nucleosomes, were more prominent. This was confirmed by densitometric measurement of DNA bands and comparison of "digestion ratios". These ratios were calculated by taking the amounts of DNA in higher order oligonucleosomes (the upper half of the third rung of the chromatin ladder, and all rungs above including the high molecular weight DNA smear), and dividing by that in the lower order oligonucleosomes (the first and second rungs, and the lower half of the third). Ionic sensitivity of the nuclease activity in T2 and T3 nuclei prepared from cells grown at both serum concentrations was confirmed. In contrast, T1 showed no endonuclease activity at low serum, whereas M8 demonstrated marked nuclease activity at high serum and a further increase at low serum (figure 3.15C).

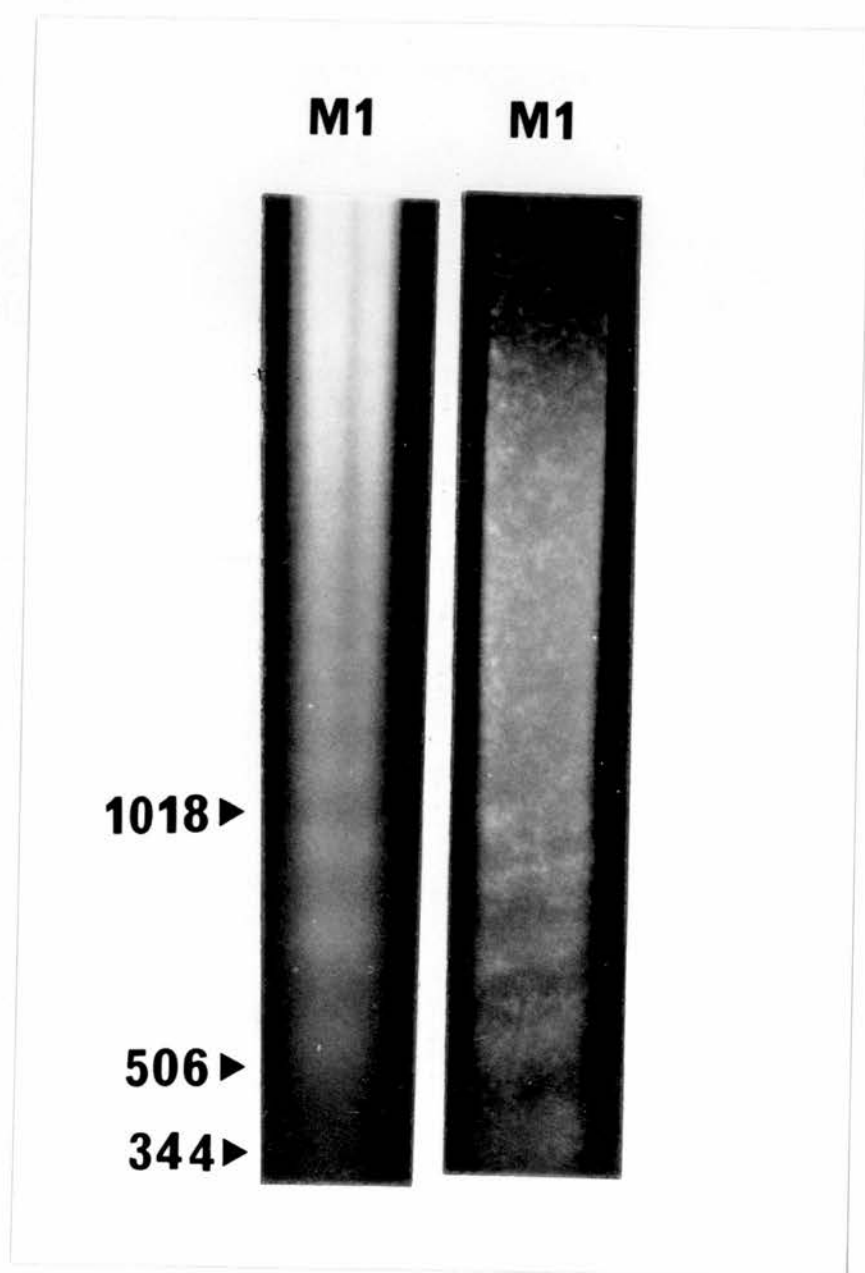


Figure 3.15

(A) Agarose gel electrophoresis of 2 DNA samples extracted from apoptotic bodies released by M1 transfectants. This shows the chromatin ladder pattern typical of apoptosis.

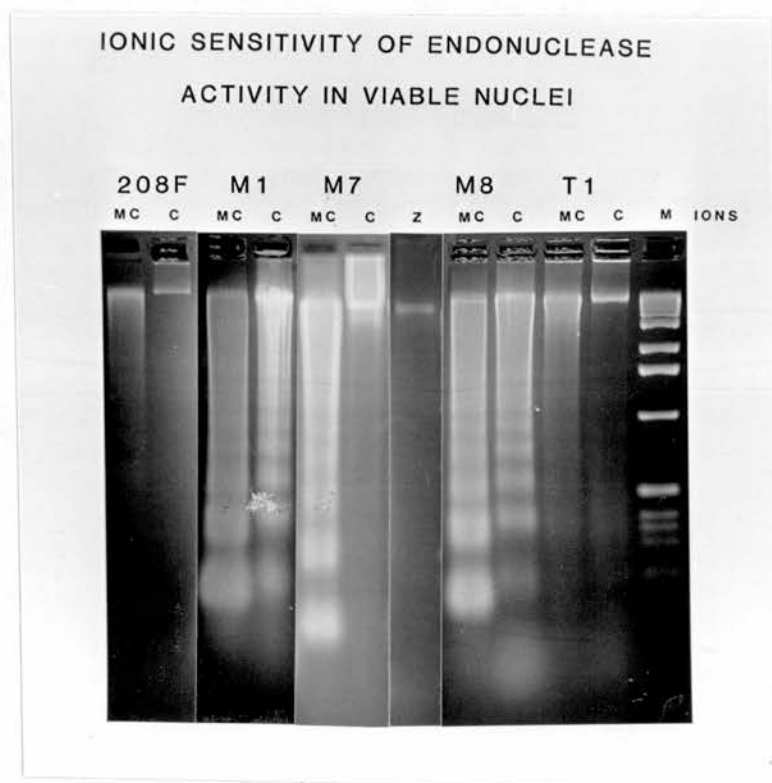


Figure 3.15

(B) Ionic sensitivity of endogenous endonuclease activity within nuclei prepared from viable cells. This shows DNA extracted from nuclei following auto-digestion in the presence or absence of magnesium and calcium ions (MC for both, C for calcium only). The parent 208F shows no chromatin digestion, the three *c-myc* transfectants, M1, M7 and M8, show chromatin laddering which is more marked when both cations are present. M8 chromatin digestion is completely inhibited by the presence of zinc ions (in the presence of magnesium and calcium ions). Samples from the *ras* transfectant T1 show no endonuclease activity.



SERUM SENSITIVITY OF ENDONUCLEASE  
ACTIVITY IN VIABLE NUCLEI

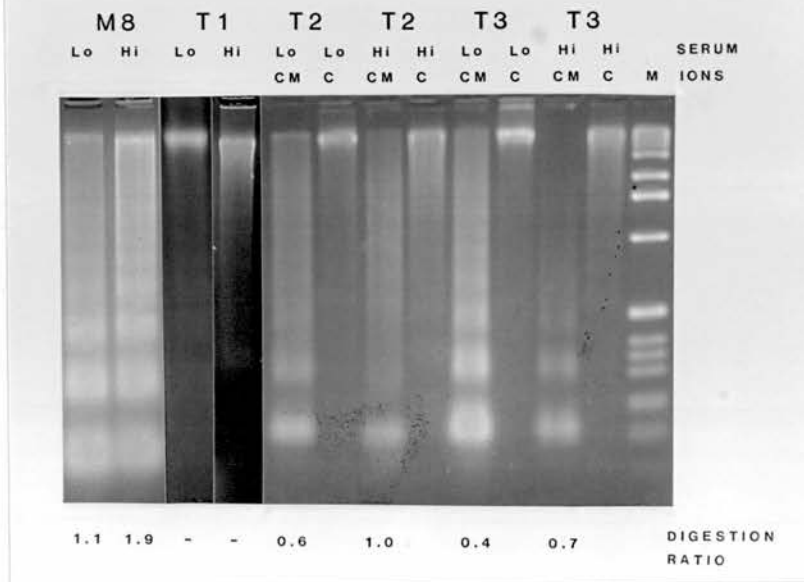


Figure 3.15

(C) Serum sensitivity of endogenous endonuclease activity in viable nuclei using the auto-digestion assay. Cells were grown for 4 hours in either low (0.05% HINCS) or high (10% HINCS) serum prior to analysis. Digestion ratio data (see table 3.5) are quoted under the appropriate track for each cell line tested at either low or high serum (for assays performed in the presence of both calcium and magnesium ions). M8 shows digestion of chromatin into oligonucleosomal fragments at both serum concentrations, with a slight increase in digestion to lower order oligonucleosomes at low serum levels indicated by the decrease in digestion ratio. T1 shows no significant chromatin digestion at either high or low serum. The tracks for the remaining 2 *ras* transfectants T2 and T3 show a pattern of chromatin digestion which differs according to the serum concentration as well as the ionic content of the assay solution. There is considerably greater chromatin digestion in the presence of both calcium and magnesium compared with calcium ions alone. Digestion patterns illustrate that both T2 and T3 show low levels of chromatin digestion, mostly into the higher order oligonucleosomes, at high serum, (measured as higher digestion ratios). In contrast, at low serum there is greater digestion into lower order oligonucleosomes (measured as reduced digestion ratios). The overall pattern is one of serum sensitive expression of endonuclease activity in T2 and T3. One kb ladder marker tracks (M) are included in figures B and C, and the positions of marker track bands are indicated in figure A. These confirm chromatin cleavage into DNA fragments that are multiples of the length of DNA wrapped around a single oligonucleosome (180 - 200 bp).



TABLE 3.5

ENDOGENOUS ENDONUCLEASE ACTIVITY AT HIGH AND LOW SERUMS

CELL LINE	HIGH SERUM (10%)			LOW SERUM (0.05%)			HI DR LO DR
	HIGHER O-N DNA	LOWER O-N DNA	DIGESTION RATIO	HIGHER O-N DNA	LOWER O-N DNA	DIGESTION RATIO	
T1	-	-	-	-	-	-	-
T2	0.203	0.195	1.04	0.140	0.226	0.62	1.68
T3	0.133	0.202	0.66	0.106	0.268	0.40	1.67
M8	0.248	0.127	1.951	0.198	0.175	1.13	1.73

Note: Cells were grown at high or low serum for 4 hours prior to analysis by the autodigestion assay for endonuclease activity. Chromatin cleavage was shown by agarose gel electrophoresis. Densitometric measurement of DNA bands allowed comparison of the "digestion ratio" (DR) for each cell line tested. The DR numerator was the amount of DNA in higher order oligonucleosomes (HIGHER O-N DNA) which included the upper half of the third rung of the chromatin ladder, and all rungs above, along with the high molecular weight DNA smear. The DR denominator was the amount of DNA in the lower order oligonucleosomes (LOWER O-N DNA) that comprised the first and second rungs, and the lower half of the third. The ratio of high serum DR (HI DR) to low serum DR (LO DR) gives an indication of the increase in chromatin digestion following the switch from high to low serum. Serum sensitivity of the nuclease activity in M8, T2 and T3 nuclei is confirmed. In contrast, T1 showed no endonuclease activity at either high or low serum.

### 3.5.1.3 CELL GROWTH KINETICS

#### 3.5.1.3.A CELL POPULATION EXPANSION

The rates of expansion of the total number of cells in growing populations were plotted as expansion curves (figures 3.16). The total number of cells after 72 hours growth and the gradients of the curves at 24 - 48 hours, were related not only to the rate of population expansion, but also the seeding efficiency and residual contact inhibition of each cell line. Therefore, the ratio of the mean cell number at 48 and 24 hours was calculated as a single measure of the proportional increase in cell number over one day, in order to compare the rates of population expansion (PE) between different cell lines whilst in maximal growth phase.

##### (i) ONCOGENE TRANSFECTANTS

The parent cell line 208F gave a PE value of 1.53, by comparison T1 (3.56), T2 (3.67) and MT7 (4.06) demonstrated higher PE rates, whereas M8 (1.20) and MT4 (1.44) showed lower values than 208F, with M7 (1.54) very similar to 208F. The PE values for M1 (2.89), T3 (1.77), MT9 (1.67) and MT10 (2.30) were intermediate (figure 3.17 and table 3.6). No statistical analysis was applied to these results as the 24 and 48 hour cell numbers were the means of only three counts.

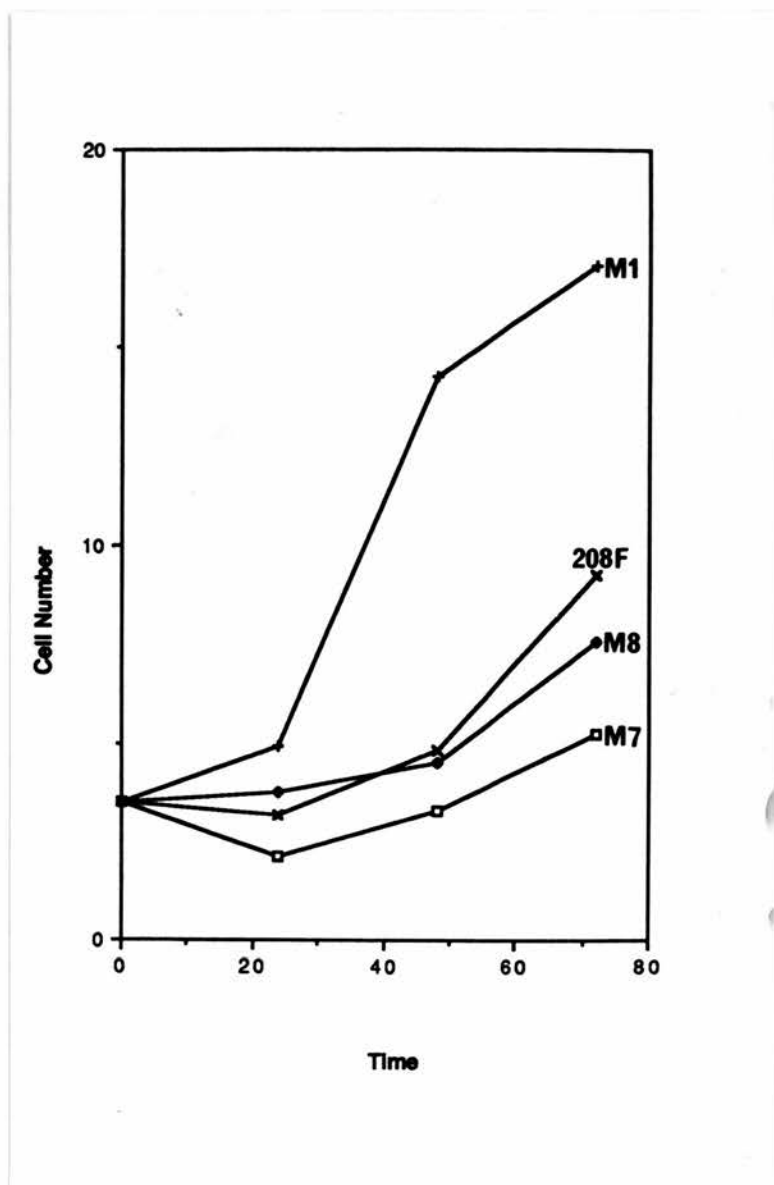


Figure 3.16

Population expansion curves are shown, which represent the net rates of growth in culture for oncogene transfectants.  $3 \cdot 10^5$  cells were seeded at time 0, and total cell numbers were counted at 24, 48 and 72 hours. (A) The three *c-myc* transfectants, M1, M7 and M8 are compared to the parent 208F. M7 and M8 show similar or slightly slower growth than that of the parent, whereas M1 appears to grow faster.

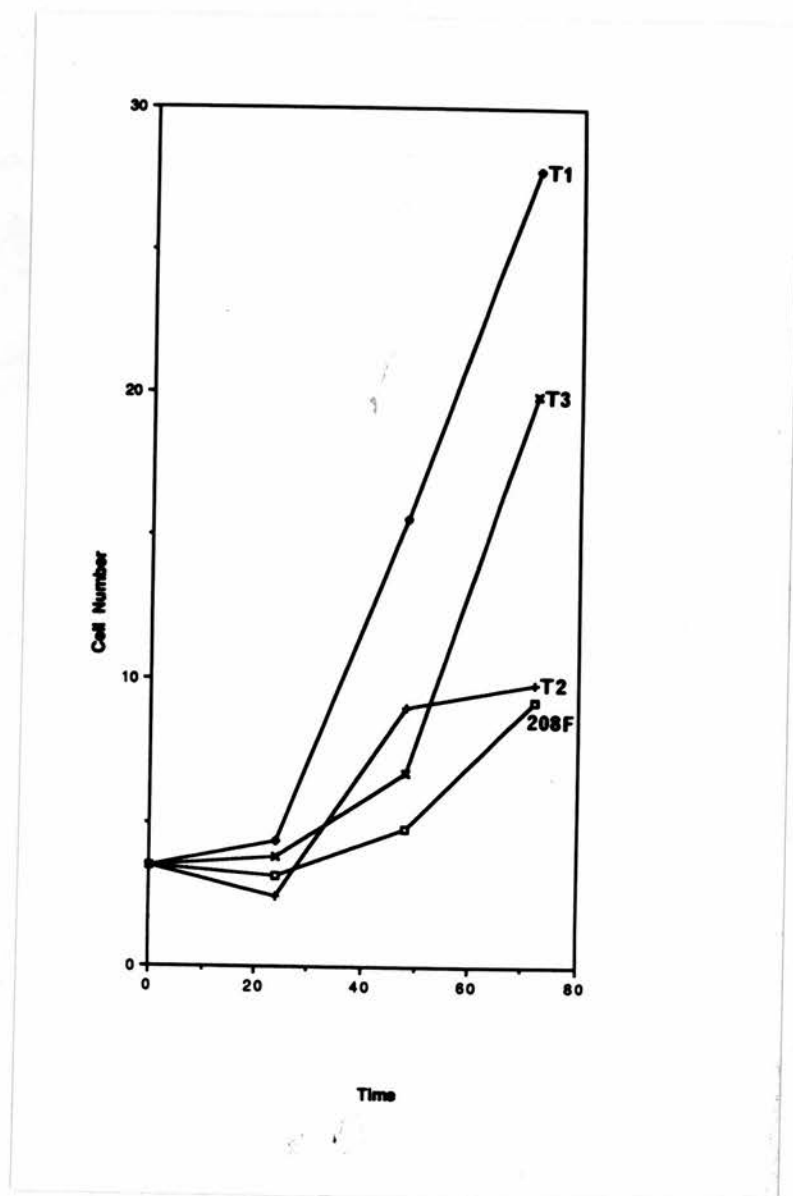


Figure 3.16

(B) Comparison of the population expansion curves for the 3 ras transfectants T1, T2, and T3 and the parent 208F. All transfectants show faster growth curves than the parent, with T1 having the steepest growth curve and highest final cell numbers.

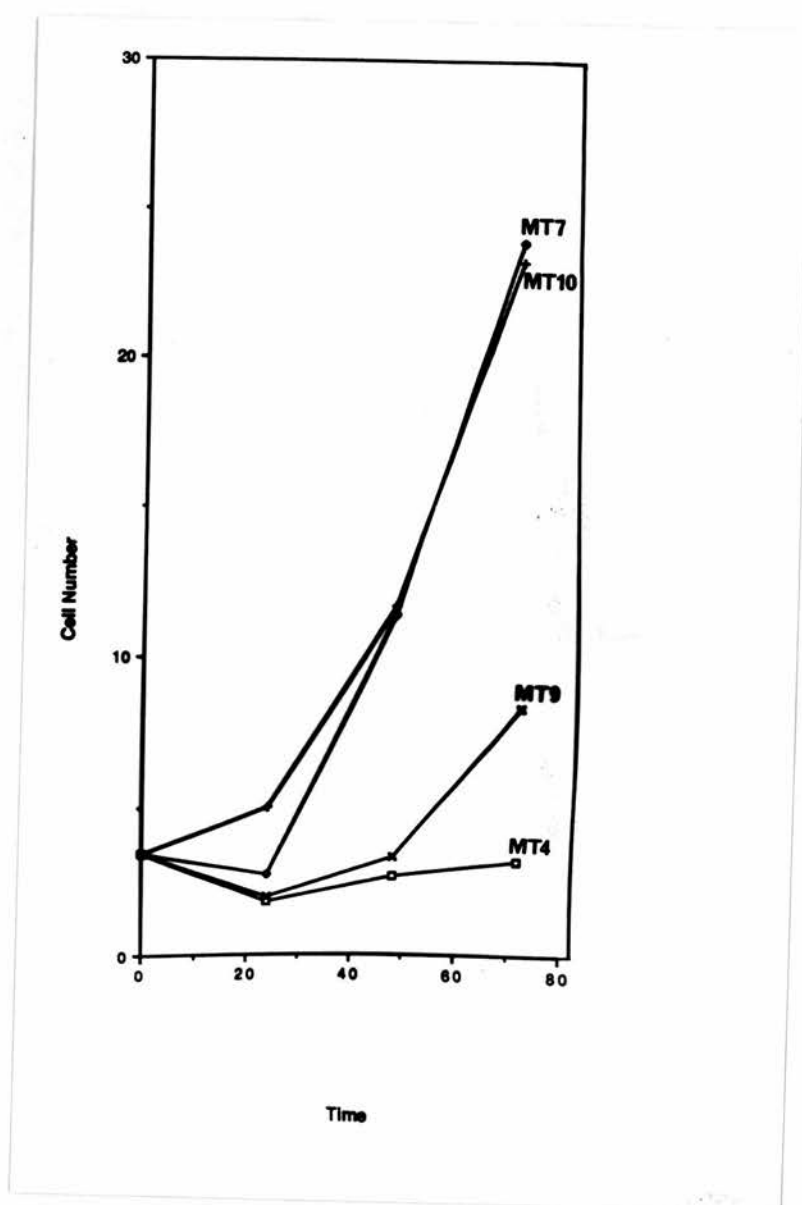


Figure 3.16

(C) Comparison of the population expansion curves for the 4 co-transfectants MT4, MT7, MT9 and MT10. MT7 and MT10 show the steepest gradients of their growth curves whereas MT9 is intermediate and MT4 has a slow growth curve similar to the myc transfectants M7 and M8.

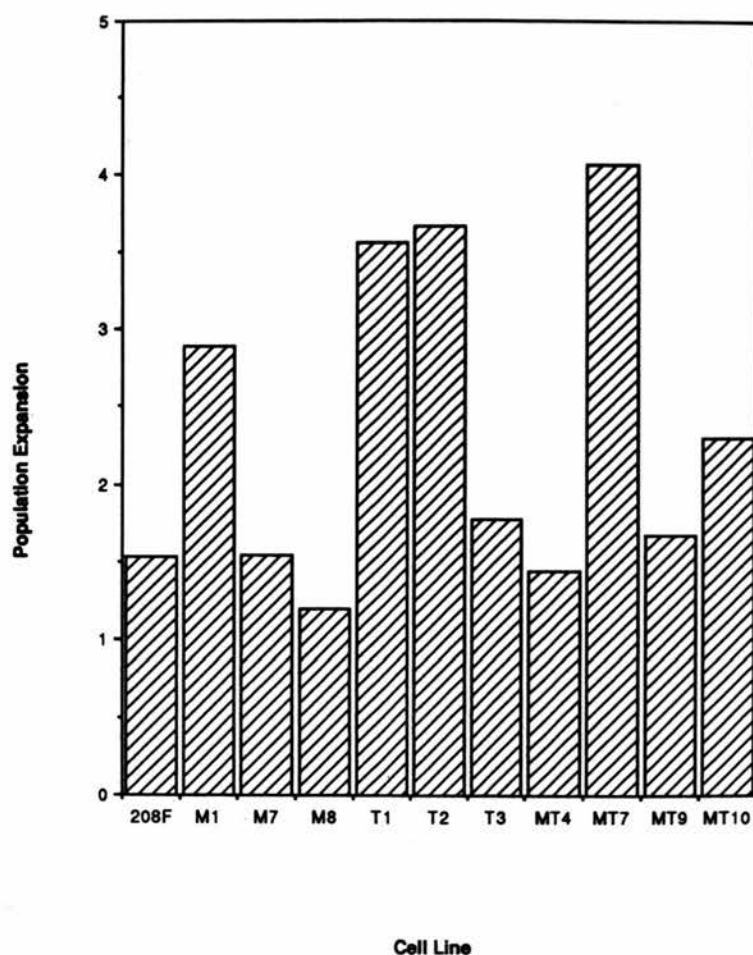


Figure 3.17

Bar chart of population expansion rates (N48/N24) for the parent 208F and 10 oncogene transfectants.

**TABLE 3.6 TRANSFECTANTS' GROWTH PARAMETERS IN VITRO AND IN VIVO**

CELL LINE	PROLIF. IND.	POP. EXP.	APO. IND. (log)	TUM. SIZE	TUM. MITO.	TUM. APO.	M/A RATIO (log)	A/M RATIO (log)	TUM. NEC.
208F	35.6	1.53	2.62(0.42)	0.29	1.03	2.8	0.44(-0.36)	3.36(0.53)	0
M1	40.4	2.89	4.57(0.66)	0.37	3.4	7.3	0.50(-0.30)	2.92(0.47)	1.7
M7	29.8	1.54	41.52(1.62)	0.51	9.6	22.6	0.50(-0.30)	3.18(0.50)	13.3
M8	39.9	1.20	15.44(1.19)	0.13	1.95	12.9	0.16(-0.80)	7.47(0.87)	5.0
T1	40.1	3.56	0.77(-0.11)	2.20	18.2	3.9	5.3(0.72)	0.22(-0.66)	31.7
T2	38.9	3.67	1.83(0.26)	2.25	18.2	11.6	1.9(0.28)	0.64(-0.20)	21.7
T3	36.6	1.77	8.44(0.93)	2.20	6.35	11.1	0.7(-0.15)	1.89(0.28)	14.2
MT4	39.4	1.44	4.10(0.61)	4.65	11.7	40.3	0.3(-0.52)	3.52(0.55)	9.2
MT7	33.1	4.06	0.62(-0.21)	2.42	26.1	4.1	6.8(0.83)	0.17(-0.77)	40.0
MT9	43.7	1.67	5.95(0.78)	1.02	12.2	3.6	3.5(0.54)	0.30(-0.52)	12.5
MT10	40.8	2.30	2.30(0.36)	1.42	16.1	10.9	1.6(0.20)	0.69(-0.16)	16.7
H16	34.7	2.19	1.28(0.11)	0.41	12.8	20.3	0.73(-0.14)	1.77(0.25)	26.7
H16R	31.9	1.52	1.22(0.09)	0.99	14.9	6.2	3.14(0.50)	0.48(-0.32)	28.3
H18	42.9	1.71	1.56(0.19)	1.05	16.7	11.5	1.50(0.18)	0.69(-0.16)	17.5
H18R	34.7	3.71	0.74(-0.13)	0.92	17.0	3.1	5.77(0.76)	0.19(-0.73)	21.7

Note: PROLIF.IND.= proliferative index (ave. 5-10 expts); POP. EXP. = population expansion rate (N48/N24) (ave. 3-9 expts); APO.IND.= apoptotic index (ave. 5-10 expts); TUM. SIZE = tumour size (3 dimensions multiplied together) (ave. 4-10 expts); TUM. MITO. = tumour mitotic counts (ave. 60 high power fields); TUM. APO. = tumour apoptotic counts (ave. 60 high power fields); M/A (mitotic/apoptotic) and A/M (apoptotic/mitotic) ratios were calculated by dividing counts/10 high power fields for each tumour (ave. 6 tumours); TUM.NEC. = tumour necrosis assessed semiquantitatively using an arbitrary scale (ave. 6 tumours).

(ii) HPV AND ONCOGENE TRANSFECTANTS

Similar values for population expansion were observed for three of the HPV containing transfectants: H16 had a PE of 2.2, H18 1.7, and H16R 1.5 (figure 3.18 and table 3.6). The only statistically significant difference found between these three values was in comparing H16 with H16R ( $p=0.032$ , student's t test on 8-9 experiments) (table 3.7A). In contrast, H18R had the substantially higher value for population expansion of 3.7 ( $p=0.0007$  for comparison with both H16 and H18, and  $p < 0.00001$  compared with H16R; student's t test on 8-9 experiments for each cell line).



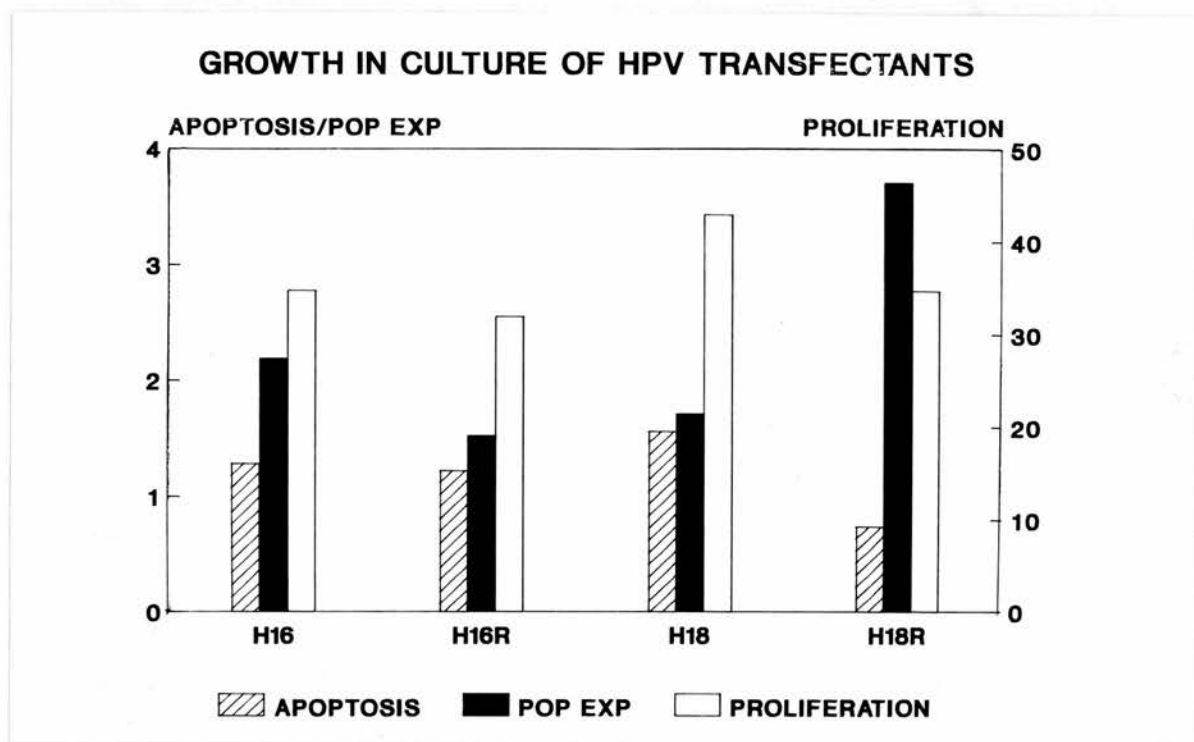


Figure 3.18

Bar chart of the growth parameters in culture of the four HPV transfectants H16, H16R, H18 and H18R. This allows comparison of the population expansion rates ( $N_{48}/N_{24}$ ), the apoptotic indices and the proliferation indices. Three lines, H16, H16R and H18 have similar overall profiles for these three parameters, but H18R differs with higher population expansion and lower apoptosis.

**TABLE 3.7A****POPULATION EXPANSION: RELATIVE COMPARISON BY STUDENT'S T TEST**

	16	16R	18	18R
16R	0.032	—		
18	NS	NS	—	
18R	0.0007	< 0.00001	0.0007	—

**TABLE 3.7B****CELL PROLIFERATION: RELATIVE COMPARISON BY STUDENT'S T TEST**

	16	16R	18	18R
16R	NS	—		
18	NS	0.012	—	
18R	NS	NS	NS	—

**TABLE 3.7C****APOPTOSIS: RELATIVE COMPARISON BY STUDENT'S T TEST**

	16	16R	18	18R
16R	NS	—		
18	NS	NS	—	
18R	NS	NS	0.028	—

NOTES: p values quoted  
NS = not significant

### 3.5.1.3.B CELL PROLIFERATION

Cell cycle analysis was performed by flow cytometric measurement of the nuclear DNA content using cells growing in the maximal growth phase for each cell line. Two computer software programs, SFITS and PARA1, were used to determine the proportion of cells in each phase of the cell cycle. The proliferative index in culture was calculated as the proportion of cells in S plus G2/M phases of the cell cycle (growth fraction). This measures the proportion of actively proliferating cells in each sample.

Several control experiments were carried out to confirm the validity of the cell preparation protocol and flow cytometric analysis.

a. For five of the cell lines, one cell sample was separated into two parts each of which was prepared separately. The average co-efficient of variance (CV) comparing the two separately prepared samples for each cell line was 7.11. This indicated that the preparation technique was reproducible.

b. Nine of the cell lines were grown in two separate flasks in parallel, and these were prepared and analysed separately. The average CV in this case was 8.35, indicating a reproducible similarity between two samples from the same cell line.

c. Two samples of three of the cell lines were grown and

prepared separately, but one sample from each pair did not have the medium replaced 4 hours before harvesting. The CV in this case was 9.19, indicating that growth medium replacement was not absolutely necessary to eliminate variation in the results.

(i) ONCOGENE TRANSFECTANTS

The proliferative indices for the parent cell lines, 208F (35.6), and the ten oncogene transfected cell lines, M1 (40.4), M7 (29.8), M8 (39.9), T1 (40.1), T2 (38.9), T3 (36.6), MT4 (39.4), MT7 (33.1), MT9 (43.7) and MT10 (40.8), were determined using SFITS computer program analysis of cell cycle data from 5-8 experiments (figure 3.19 and table 3.6). These were statistically analysed by the ANOVA test, which showed no significant differences ( $F=1.38$ ,  $v_1=10$ ,  $v_2=75$ ). Paired comparisons by the student's t test confirmed the absence of statistically significant differences, with one exception: M7 and MT9 ( $p=0.02$ ) (table 3.8). Cell cycle analysis by PARAl computer software produced similar results with no significant differences by ANOVA testing ( $F=0.74$ ) or student's t testing.

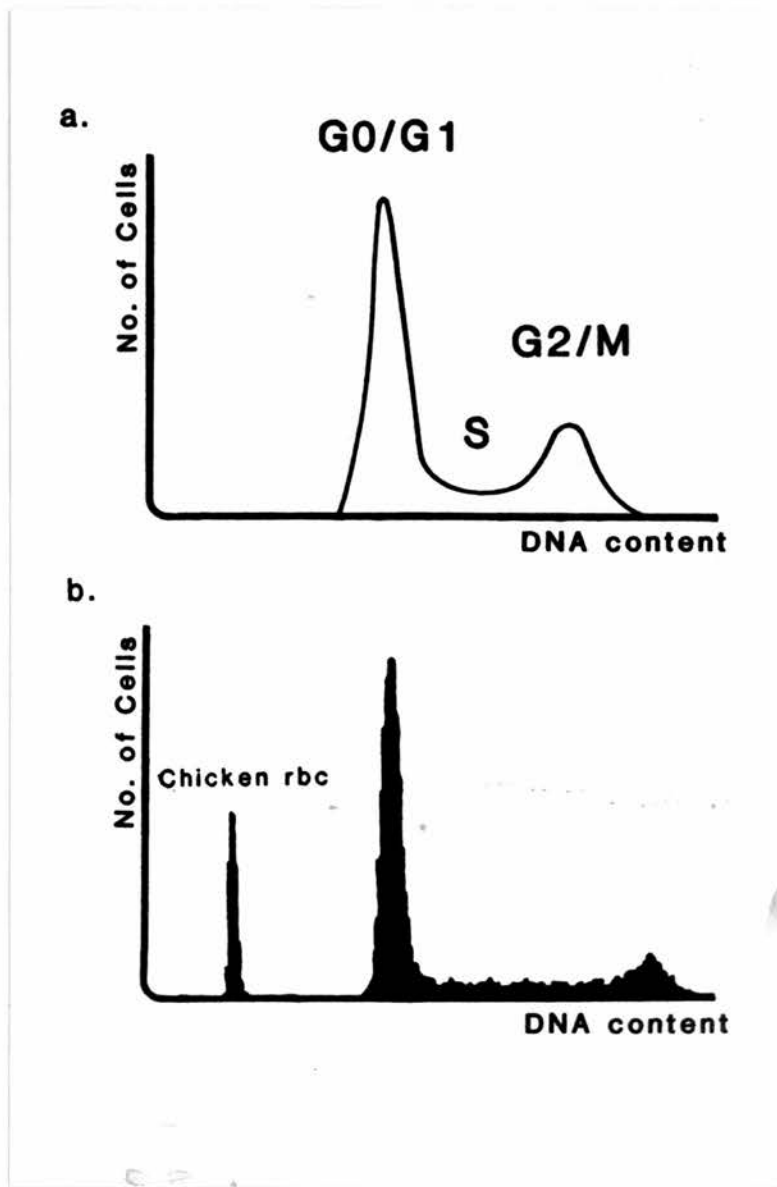


Figure 3.19

Flow cytometric analysis of the proportion of proliferating cells in log phase cultures.

(A) Theoretical graph of DNA content against number of cells (a) shows two major peaks. The first peak contains cells with the normal DNA content (G0/G1), the second peak cells about to undergo division (G2/M), with the S phase proportion of cells lying between the two peaks. Actual DNA histogram from flow cytometric analysis of the M1 cell line (b). The histogram shows the two major peaks, and chicken red blood cells which are included as a DNA content standard (approximately 35% of the DNA content of the fibroblast G0/G1 peak).

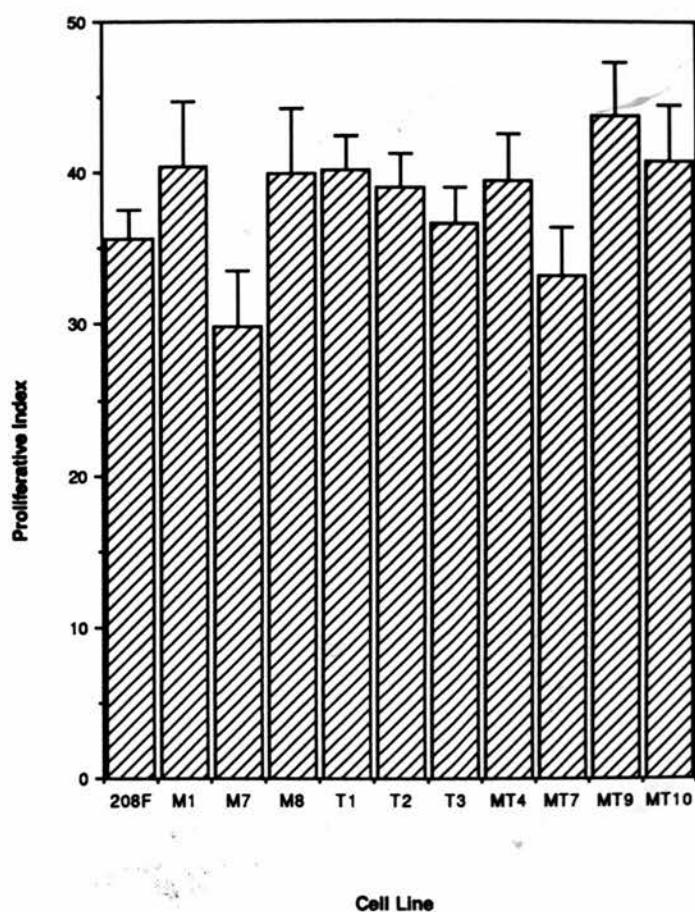


Figure 3.19

(B) Bar chart of proliferative indices (S+G2/M) of parent 208F and 10 oncogene transfectants (mean  $\pm$  SEM), following analysis of DNA histograms with SFITS computer program.

TABLE 3.8

PROLIFERATIVE INDICES OF ONCOGENE TRANSFECTANTS: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10
M1	NS	—									
M7	NS	NS	—								
M8	NS	NS	NS	—							
T1	NS	NS	NS	NS	—						
T2	NS	NS	NS	NS	NS	—					
T3	NS	NS	NS	NS	NS	NS	—				
MT4	NS	NS	NS	NS	NS	NS	NS	—			
MT7	NS	NS	NS	NS	NS	NS	NS	NS	—		
MT9	NS	NS	0.02	NS	NS	NS	NS	NS	NS	—	
MT10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	—

NOTES: p values quoted; NS = not significant. SFITS software used for analysis of cell cycle data.

The proportion of cells in S + G2/M phases were added to provide the proliferative index.

ANOVA test on same data: F = 1.38, not significant.

## (ii) HPV AND ONCOGENE TRANSFECTANTS

There were similar proliferation index values for the four cell lines H16 (34.7), H16R (31.9), H18 (42.9) and H18R (34.7) (figure 3.18 and table 3.6). The only significant difference was between H18 and H16R ( $p = 0.012$ , student's t-test on 8-9 experiments) (table 3.7B).

### 3.5.1.3.C CELL DEATH BY APOPTOSIS

#### (i) ONCOGENE TRANSFECTANTS

The apoptotic indices (AI) were spread over a wide range (table 3.6). M7 (41.52) and M8 (15.44) had very high apoptotic indices, whereas T1 (0.77), and MT7 (0.62) had very low values. The value for T2 (1.83) was lower than that of the parent cell line 208F (2.62), and the remaining cell lines had intermediate apoptotic indices: M1 (4.57), T3 (8.44), MT4 (4.10), MT9 (5.95) and MT10 (2.30) (figure 3.20). The apoptotic index values were log-transformed and both the linear and log data were analysed using the ANOVA test, which indicated significant variation between the cell lines in both cases ( $p < 0.0001$ ). AI values, both linear and log, for individual pairs of cell lines were compared by the student's t test which showed statistically significant differences for most comparisons; in particular, M7 and M8 were significantly higher, and T1 and T2 were significantly lower than most of the other cell lines (table 3.9).



(ii) HPV AND ONCOGENE TRANSFECTANTS

The moderate levels of apoptotic index for the three transfectants H16 (1.28), H18 (1.56) and H16R (1.22) were similar, with no significant differences by the student's t test. However, the very low value for H18R (0.74) was similar to that for T1 (0.77), and was significantly different from the value for H18 ( $p = 0.028$ , student's t-test on 8-9 experiments) (figure 3.18, and tables 3.6 and 3.7).

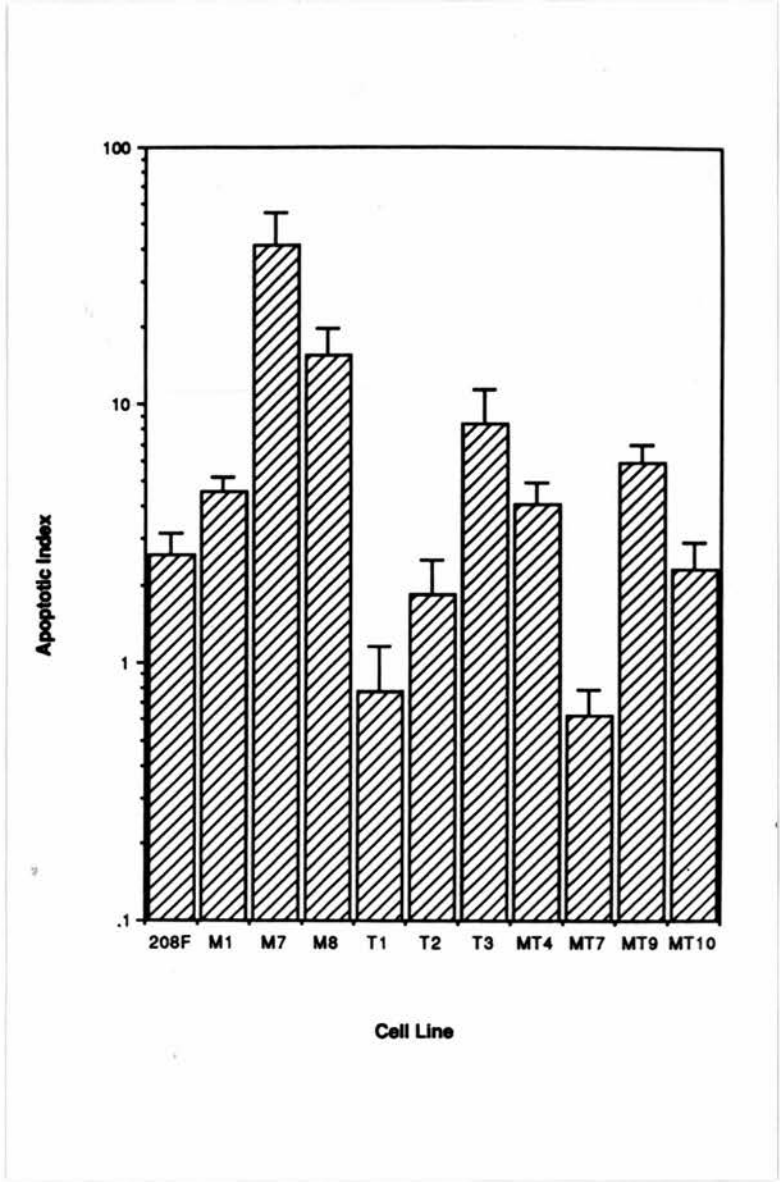


Figure 3.20

Bar chart of the mean apoptotic index  $\pm$  SEM for the parent 208F and 10 oncogene transfectants.

TABLE 3.9A LINEAR DATA

APOPTOTIC INDICES OF ONCOGENE TRANSFECTANTS: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10
M1	0.039	—									
M7	0.039	0.046	—								
M8	0.046	NS	NS	—							
T1	0.02	0.008	0.003	0.03	—						
T2	NS	0.013	0.036	0.039	NS	—					
T3	NS	NS	NS	NS	NS	NS	—				
MT4	NS	NS	0.044	NS	0.013	NS	NS	—			
MT7	0.015	0.0015	0.033	0.029	NS	NS	NS	0.011	—		
MT9	0.023	NS	NS	NS	0.0032	0.0091	NS	NS	0.0036	—	
MT10	NS	0.028	0.038	0.043	NS	NS	NS	NS	0.045	0.016	—

NOTES: P values quoted

NS = not significant

**TABLE 3.9B LOG TRANSFORMED DATA**  
**APOPTOTIC INDICES OF ONCOGENE TRANSFECTANTS: RELATIVE COMPARISONS BY STUDENT'S T TEST**

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10
M1	0.043	—									
M7	<0.00001	0.0002	—								
M8	0.0028	0.019	0.05	—							
T1	0.015	0.0043	<0.00001	0.0006	—						
T2	NS	0.022	<0.00001	0.0013	NS	—					
T3	NS	NS	0.01	NS	0.0027	0.02	—				
MT4	NS	NS	0.0002	0.012	0.0071	NS	NS	—			
MT7	0.0004	<0.00001	<0.00001	<0.00001	NS	NS	0.0015	0.0004	—		
MT9	0.0063	NS	0.0005	0.047	0.0025	0.011	NS	NS	<0.00001	—	
MT10	NS	0.042	<0.00001	0.0018	0.03	NS	0.043	NS	0.0076	0.014	—

NOTES: p values quoted  
 NS = not significant.

### 3.5.2 TUMOUR GROWTH IN IMMUNE SUPPRESSED MICE

All T24-ras containing transfectants formed tumours in every mouse injected, whereas 2 out of 11 mice injected with M1, 1 out of 7 with M7, and 1 out of 7 with M8 (14%-18%) failed to form primary tumours. The parent cells (208F) formed indolent nodules in 10 out of 11 mice, and these did not increase in size. Histological analysis revealed that although some of the 208F cells mingled with subcutaneous adipocytes, there was no evidence of invasion of skin or muscle. There was a small amount of 208F cell turnover, in that low counts of mitotic and apoptotic figures were seen within these nodules (see below). Simple calculations, based on estimation of 208F cell volume and the volume of the nodule, showed that the numbers of 208F cells must have been of the same order of magnitude at the end of the 12 day assay period, as in the inoculum, confirming the non-progressive and non-malignant nature of these nodules.

#### 3.5.2.1 TUMOURS FORMED BY ONCOGENE TRANSFECTANTS

Sizes of the primary tumours formed by the 3 T24-ras transfected cell lines T1 (2.2), T2 (2.24), and T3 (2.2) were greater than the 3 cell lines transfected with the c-myc oncogene M1 (0.37), M7 (0.51), and M8 (0.13), and also the nodules formed by the parent cell line 208F (0.29) (table 3.6 and figure 3.21).

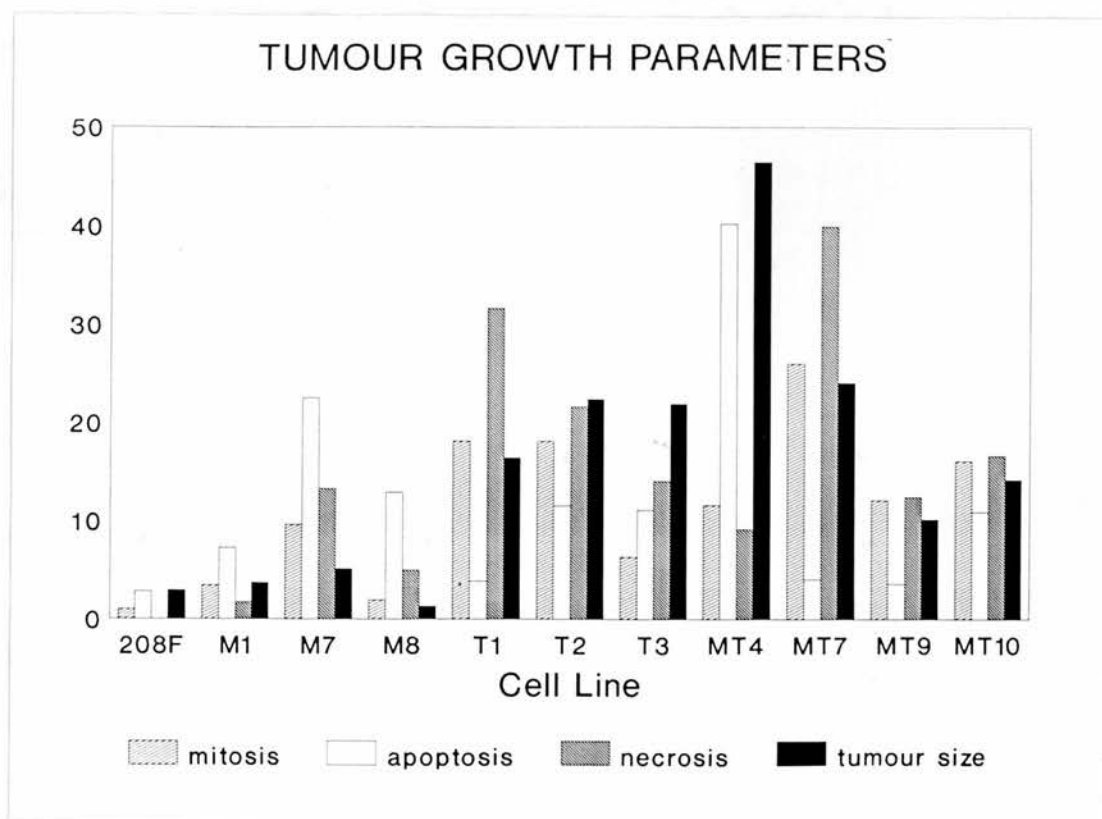


Figure 3.21

Growth properties of tumours derived from the parent 208F and 10 rat fibroblast cell lines transfected with *c-myc* and/or *T24-ras*. Mean tumour sizes (3 dimensions multiplied together) are shown after 12 days growth in immune suppressed mice injected with  $10^7$  cells. Oncogene transfectants generated progressively enlarging invasive malignant tumours, whereas 208F formed non-progressive, indolent nodules. Mitosis and apoptosis within apparently viable regions of tumours were scored as figures per 10 high power fields. Necrosis was assessed semiquantitatively using an arbitrary scale. The *T24-ras* containing cell lines formed the largest tumours, with much necrosis, which displayed mostly high mitotic but low apoptotic rates. In contrast, the *c-myc* transfectants generated smaller tumours, with little necrosis, that showed markedly higher rates of apoptosis relative to those for mitosis.

TABLE 3.10A

## TUMOUR SIZE: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10
M1	NS	—									
M7	NS	NS	—								
M8	NS	NS	0.012	—							
T1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	—						
T2	0.0047	0.0043	0.0077	0.0029	NS	—					
T3	0.0015	0.001	0.0026	0.0008	NS	NS	—				
MT4	0.0002	0.0001	0.0003	0.0002	0.004	0.0059	0.0044	—			
MT7	0.0001	0.0007	0.0005	0.0001	0.0039	NS	NS	0.0013	—		
MT9	0.0008	0.013	0.0088	0.0005	0.0001	0.031	0.014	0.0007	NS	—	
MT10	0.0013	0.0029	0.0049	0.0009	0.015	NS	NS	0.0011	NS	NS	—

NOTES: p values quoted

NS = not significant

**TABLE 3.10B****TUMOUR SIZE: RELATIVE COMPARISONS BY STUDENT'S T TEST**

	208F	16	16R	18	18R
16	NS	—			
16R	0.0001	0.0037	—		
18	0.035	NS	NS	—	
18R	NS	NS	NS	NS	—

NOTES:     p values quoted  
               NS = not significant



For the cell lines transfected with both oncogenes, however, MT4 (4.65) produced the largest tumours, whereas MT7 (2.42), MT9 (1.02) and MT10 (1.42), produced tumours of intermediate size, compared to the 3 ras transfectants, although all were greater in size than the 3 myc transfectants and the parent cell line. The ANOVA test demonstrated statistically significant variations between these values ( $p < 0.0001$ ), and differences between particular pairs of cell lines were revealed by the student's t test (table 3.10A).

Higher amounts of tumour necrosis were observed in all transfectants containing the ras oncogene (T1 31.7, T2 21.7, T3 14.2, MT4 9.2, MT7 40.0, MT9 12.5, and MT10 16.7) compared with those containing myc (M1 1.7, M7 13.3, and M8 5.0) and the parent cell line (208F 0.0) (figure 3.21). Statistical analysis was not applied as these observations were based on subjective semiquantitative assessment of regions of necrosis.

Comparison of the relative values for cell proliferation, as measured by the mitotic count, showed that all oncogene transfected cell lines had greater values than the parent cell line 208F (1.03) (table 3.6 and figure 3.21). Two of the three T24-ras transfectants had greater mitotic counts (T1 18.2, T2 18.2) than the 3 c-myc transfectants (M1 3.4, M7 9.6, M8 1.95), and the third (T3 6.35) was similar. Three of the double oncogene transfectants containing both T24-ras and c-myc

had intermediate values (MT4 11.7, MT9 12.2, MT10 16.1), and one (MT7 26.1) was comparable with the two highest ras transfectants. ANOVA testing indicated significant variation between these values ( $p < 0.0001$ ), with significant differences between almost all comparisons of pairs of cell lines were demonstrated by the student's t test (table 3.11).

In contrast, a different pattern emerged when comparing the measures of cell death by apoptosis, as determined by the apoptotic counts. All transfected cell lines had higher levels of apoptosis than the parent cell line 208F (2.8). Overall, 2 out of 3 of the c-myc transfectants had higher measures of apoptosis (M1 7.3, M7 22.6, M8 12.9), compared with the 3 T24-ras transfectants (T1 3.9, T2 11.6, T3 11.1). The double oncogene transfectants containing both T24-ras and c-myc had similar values to the ras only transfectants, with the exception of MT4 (derived from c-myc transfectant M1) with an apoptotic count of 40.3 (MT7 4.1, MT9 3.6, MT10 10.9) (figure 3.21). There was significant variation between these values by ANOVA testing ( $p < 0.0001$ ), with significant differences revealed by the student's t test for most pairs of cell lines compared (table 3.12).

TABLE 3.11

## TUMOUR MITOTIC COUNTS: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10	16	16R	18	18R
M1	*	—													
M7	*	*	—												
M8	0.0011	0.0018	*	—											
T1	*	*	*	*	—										
T2	*	*	*	*	NS	—									
T3	*	*	0.001	*	*	*	—								
MT4	*	*	0.037	*	*	*	*	—							
MT7	*	*	*	*	*	*	*	*	—						
MT9	*	*	0.011	*	*	*	*	NS	*	—					
MT10	*	*	*	*	0.046	NS	*	*	*	0.0001	—				
16	*	*	0.0032	*	*	*	*	NS	*	NS	0.0011	—			
16R	*	*	*	*	0.0047	0.0064	*	0.0024	*	0.011	NS	NS	—		
18	*	*	*	*	NS	NS	*	*	*	*	NS	0.0005	NS	—	
18R	*	*	*	*	NS	NS	*	*	*	*	NS	0.0003	NS	NS	—

NOTES: p values quoted; NS = not significant; \* =  $p < 0.00001$   
60 mitotic counts analysed for each cell line.

TABLE 3.12

## TUMOUR APOPTOTIC COUNTS: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10	16	16R	18	18R
M1	*	—													
M7	*	*	—												
M8	*	*	*	—											
T1	0.046	*	*	*	—										
T2	*	0.0007	*	NS	*	—									
T3	*	0.0008	*	NS	*	NS	—								
MT4	*	*	*	*	*	*	*	—							
MT7	0.012	*	*	*	NS	*	*	*	—						
MT9	NS	*	*	*	NS	*	*	*	NS	—					
MT10	*	0.0003	*	NS	*	NS	NS	*	*	*	—				
16	*	*	NS	*	*	*	*	*	*	*	*	—			
16R	*	NS	*	*	0.005	*	*	*	0.001	*	*	*	—		
18	*	*	*	NS	*	NS	NS	*	*	*	NS	*	*	—	
18R	NS	*	*	*	NS	*	*	*	0.04	NS	*	*	*	*	—

NOTES: p values quoted; NS = not significant; \* =  $p < 0.00001$   
 60 Apoptotic counts for each cell line were analysed

The raw data for mitotic counts and apoptotic counts were combined to form ratios of mitosis/apoptosis and apoptosis/mitosis (table 3.6). The ratios (M/A and A/M) simplify the comparison of different tumour cell lines by combining parameters for cell proliferation and cell death by apoptosis in one index. It also adjusts for any bias introduced according to differences in cell size: morphological analysis of primary tumour growth shows that cell lines containing the T24-ras oncogene formed tumours with large cells of high cytological grade, whereas those containing c-myc oncogene formed tumours with smaller cells and lower cytological grade. High power fields of different tumours were likely to contain slightly different numbers of cells due to the difference in cell size and cell density, and a combination of the mitotic count divided by the apoptotic count (or vice versa) cancels out any bias due to differences in cell size between the different lines.

The means of the M/A ratios of the parent 208F and the ten oncogene transfectants showed significant variation on ANOVA testing ( $p < 0.0001$ ), with most of the paired comparisons by the student's t test revealing significant differences (table 3.13). There was a striking relationship between the mean M/A ratio and the mean tumour size for the 3 c-myc transfectants when compared with those values for the 3 T24-ras transfectants (figure 3.22). The 3 c-myc transfectants had low values for M/A ratio which were less than unity (M1 0.50, M7 0.50, M8

0.16) and also low values for tumour size (M1 0.37, M7 0.51, M8 0.13), as did the parent 208F (M/A ratio 0.44; tumour size 0.29), whereas the 3 T24-ras transfectants had higher values for the M/A ratio (T1 5.3, T2 1.9, T3 0.7) and also formed larger tumours (T1 2.2, T2 2.2, T3 2.2). The mean M/A ratios correlated with the mean tumour sizes with a value of  $r = 0.65$  (for 208F, M1, M7, M8, T1, T2 and T3). The 4 double oncogene transfectants containing both T24-ras and c-myc also showed both high M/A ratios and produced large tumours, with the exception of MT4 (derived from M1) which had an M/A ratio of 0.3 and a tumour size of 4.7 (M/A ratios: MT7 6.8, MT9 3.5, MT10 1.6; and tumour sizes: MT7 1.4, MT9 1.0, MT10 1.4). The A/M ratios were also calculated as they represent the in vivo equivalent of the apoptotic index in culture. A/M ratios for all 15 cell lines (parent 208F, 10 oncogene transfectants, and 4 HPV transfectants) showed an inverse correlation with tumour sizes of correlation coefficient  $r = - 0.63$  (table 3.6).

TABLE 3.13

## MITOSIS/APOPTOSIS RATIO: RELATIVE COMPARISONS BY STUDENT'S T TEST

	208F	M1	M7	M8	T1	T2	T3	MT4	MT7	MT9	MT10	16	16R	18	18R
M1	NS	—													
M7	NS	NS	—												
M8	NS	NS	NS	—											
T1	0.0034	0.0036	0.0037	0.0025	—										
T2	0.0074	0.0088	0.0076	0.0038	0.014	—									
T3	NS	NS	NS	0.022	0.0044	0.015	—								
MT4	NS	NS	NS	0.006	0.0029	0.0056	NS	—							
MT7	0.0028	0.0029	0.003	0.0023	NS	0.0097	0.0034	0.0025	—						
MT9	0.0004	0.0004	0.0005	0.0004	NS	0.014	0.0007	0.0006	0.037	—					
MT10	0.0054	0.0068	0.0065	0.0025	0.012	NS	0.016	0.004	0.0072	0.0044	—				
16	NS	NS	NS	0.02	0.0045	0.017	NS	NS	0.0035	0.0007	0.02	—			
16R	0.045	0.048	0.048	0.031	NS	NS	NS	0.037	0.039	NS	NS	NS	—		
18	0.0002	0.0003	0.001	0.0001	0.0094	NS	0.0034	0.0001	0.006	0.005	NS	0.005	NS	—	
18R	0.0015	0.0016	0.0017	0.0011	NS	0.0053	0.002	0.0013	NS	0.049	0.0053	0.002	NS	0.004	—

NOTES: p values quoted; NS = not significant;  
6 tumours for each cell line were analysed

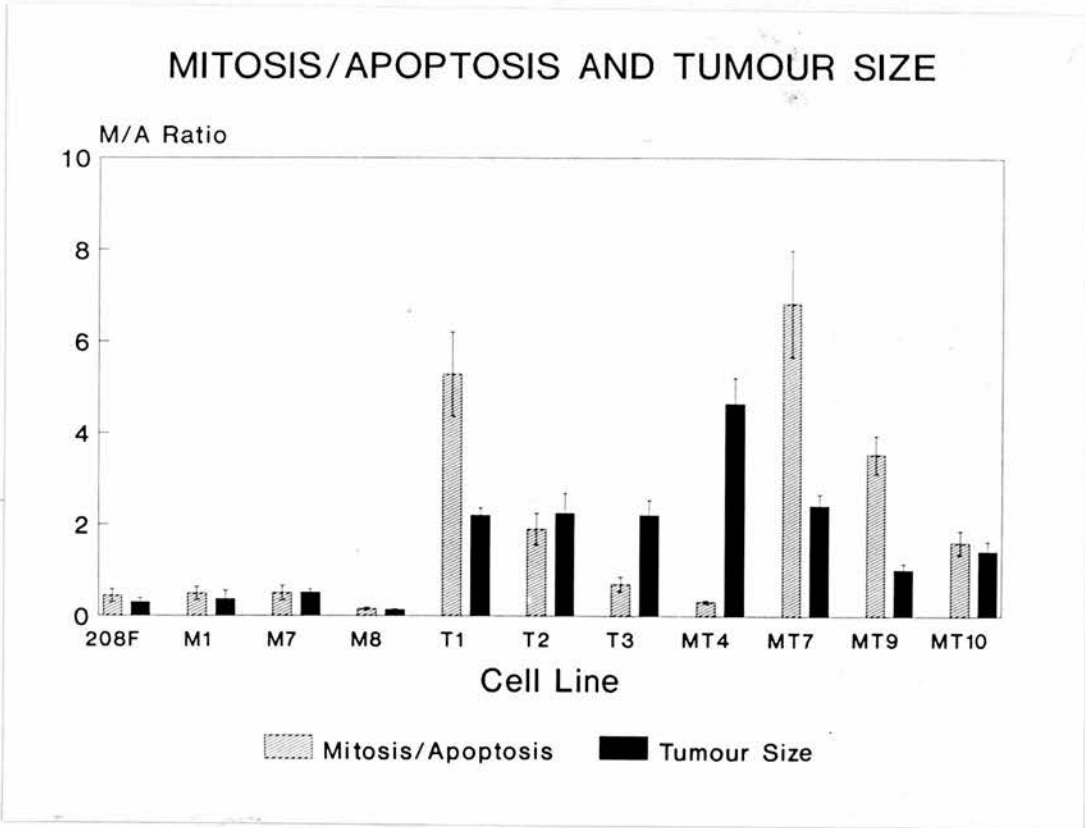


Figure 3.22

Bar chart of the mean ratios of mitosis/apoptosis  $\pm$  SEM, compared with the mean tumour sizes  $\pm$  SEM, for the parent 208F and 10 oncogene transfectants.



### 3.5.2.2 TUMOURS FORMED BY TRANSFECTANTS CONTAINING PAPILLOMAVIRUS AND RAS GENES

The tumour growth parameters were compared for the 4 transfectants H16, H18, H16R and H18R (figure 3.23 and table 3.6). All transfectants formed tumours which were larger in size than the parent cell line 208F (0.29). Tumours formed by H18 (1.05), were larger than those formed by H16 (0.41), whereas H16R (0.99) were similar in size to those formed by H18R (0.92). Student's t tests showed significant differences between 208F and both H16R ( $p=0.0001$ ) and H18 ( $P=0.035$ ); and also between H16 and H16R ( $p=0.0037$ ) (Table 3.10B).

All 4 transfectants formed tumours with similarly high levels of necrosis: H16 26.7, H16R 28.3, H18 17.5 and H18R 21.7 (figure 3.23). The values for tumour cell proliferation, as determined by the mitotic counts, were in a range from 12.8 to 17 for the 4 transfectants (H16 12.8, H16R 14.9, H18 16.7, H18R 17.0) (table 3.6 and figure 3.23). There was significant variation between them detected by the ANOVA test ( $p=0.001$ ). The student's t test showed significant differences only between H16 and H18 ( $p=0.0005$ ), and H16 and H18R ( $p=0.0003$ ) (table 3.11).

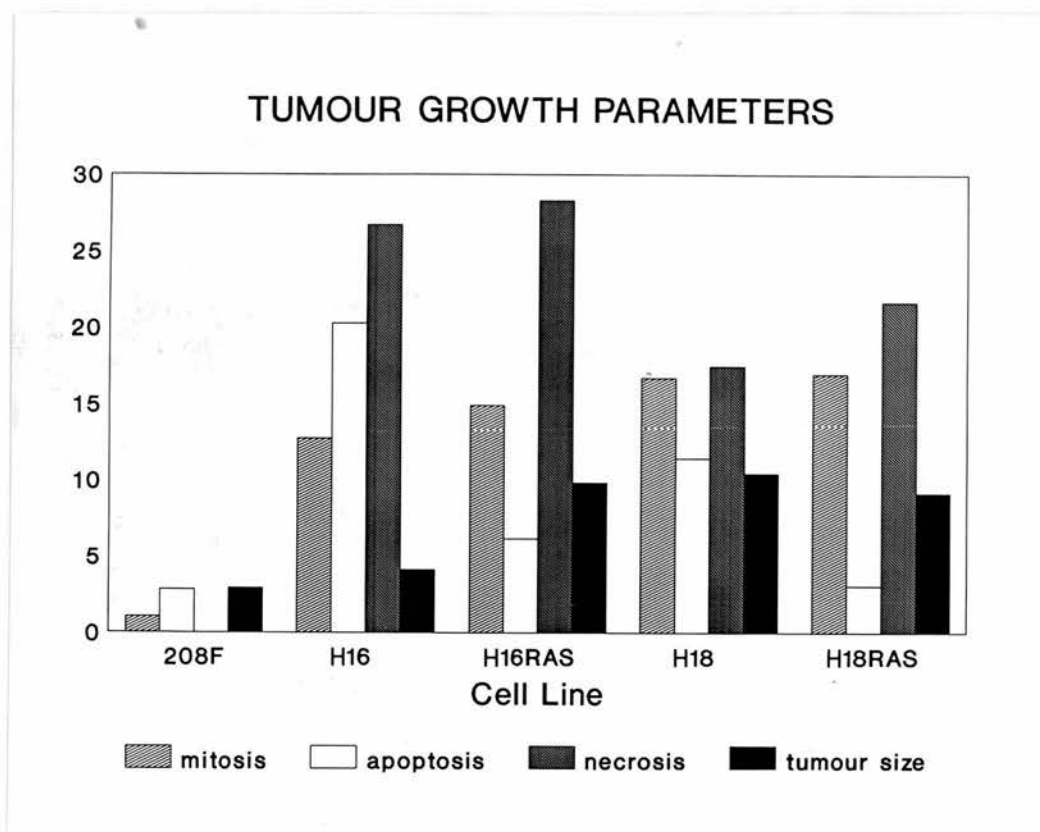


Figure 3.23

Bar chart of growth properties of tumours derived from the parent 208F and 4 HPV + ras transfectants (H16, H16R, H18, H18R). Tumour size, necrosis, mitosis and apoptosis are shown. H16R generated larger tumours than H16, but both showed high levels of necrosis. The presence of ras along with HPV 16 suppressed the levels of apoptosis whereas those of mitosis increased only slightly. H18R and H18 showed similar tumour sizes and levels of necrosis, but the presence of ras suppressed tumour cell apoptosis, whereas the levels of mitosis were almost identical.

Measures of cell death by apoptosis, as determined by apoptotic counts, varied between the 4 cell lines by ANOVA testing ( $p < 0.0001$ ), with H16 having a higher value (20.3) in comparison with the markedly smaller value for H16R (6.2). Similarly tumours formed by H18 had higher measures of apoptosis (11.5) than those formed by H18R (3.1) (figure 3.23). All paired comparisons by the student's t test of the values for these four transfectants were showed significant differences at  $p < 0.00001$  (table 3.12).

The measures of mitosis and apoptosis were combined to form M/A and A/M ratios, which showed significant variation on ANOVA testing ( $p < 0.0001$ ), with many significant differences in paired comparisons by the student's t test (tables 3.6 and 3.13). These differences were most marked between the transfectants containing HPV alone, compared with those containing both HPV and T24-ras. Tumours formed by H16 had significantly lower M/A ratios (0.73), than those generated by H18 (1.50) ( $p = 0.005$ ). The trend for larger M/A ratios in tumours formed by H16R (3.14), as compared with those of H16 and H18 was not significant. In contrast, M/A ratios in H18R tumours (5.77) were significantly larger than those in H16 tumours ( $p = 0.002$ ) and H18 tumours ( $p = 0.004$ ). M/A ratios were also compared to the sizes of tumours generated by the HPV transfectants (figure 3.24).

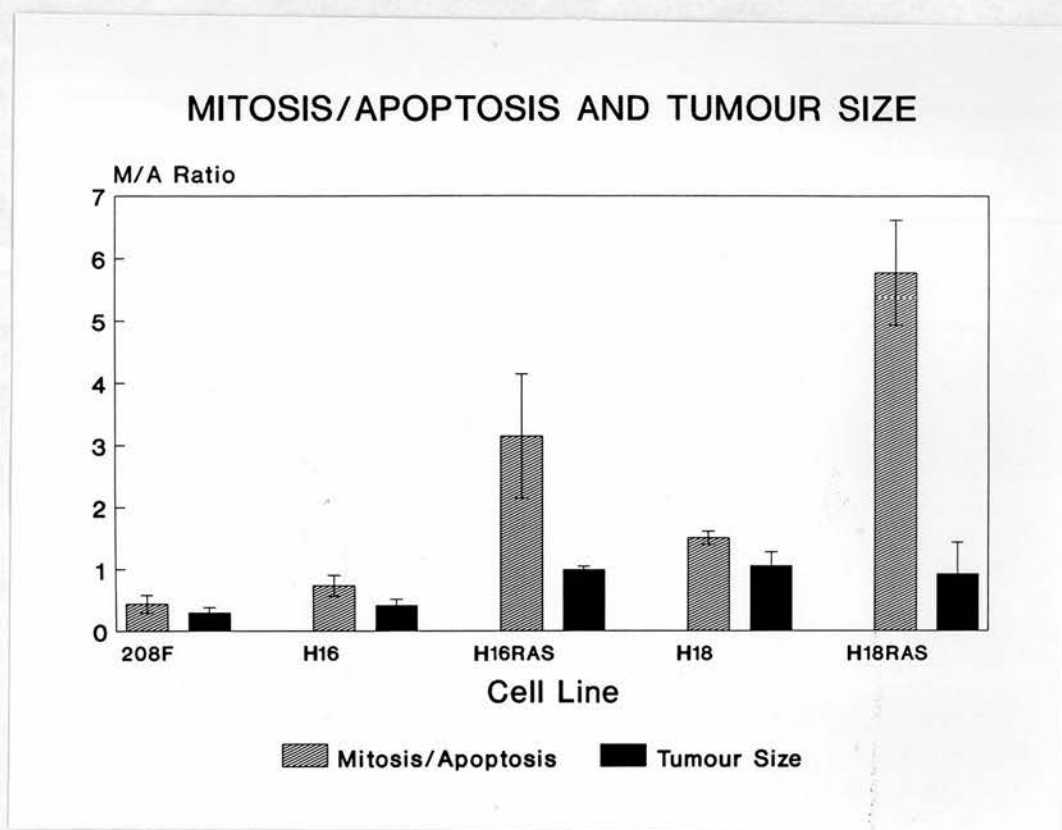


Figure 3.24

Bar chart of the ratios of mitosis/apoptosis  $\pm$  SEM, compared with the mean tumour sizes  $\pm$  SEM, for HPV transfectants. H18R has a markedly greater M/A ratio than both H16 and H18, and H18 has a higher ratio than H16.

### 3.6 DISCUSSION

#### 3.6.1 CONSERVATION AND EXPRESSION OF TRANSFECTED GENES

The presence of transfected plasmid DNA within each of the cell lines was confirmed by Southern blotting or PCR. Transcription to RNA of c-myc in M7 and M8, and T24-ras in T1, T2 and T3, was confirmed by a new assay using reverse transcription followed by PCR, validated by dot blotting for c-myc. Oncogene expression in M1 and T1 has been demonstrated previously (Spandidos, 1985; Wyllie et al, 1987). The levels of expression are not yet established, however northern blot or ELISA analysis would allow relative quantitation. Molecular and morphological data are mutually supportive, in that transformation was observed immediately following transfection.

#### 3.6.2 CLUSTERING OF PHENOTYPIC PROPERTIES OF THE TRANSFECTANTS

All cell lines differ from one another in some respects. However, some are similar in certain characteristics, and this raises questions about why such clustering of phenotypic properties should occur. Clustering may provide important insights into relationships between phenotypic properties, and their regulation by myc, ras, and HPV genes.

**TABLE 3.14 MORPHOLOGY IN CULTURE**

Topo-inhibited Polygonal	Fascicle Formation Mild Refractility	Moderate Refractility	Focus Formation Marked Refractility
208F Primary Fibroblasts	M8 M7 M1 MT4	H16 H18 MT9 MT10 T3	T1 T2 MT7 H16R H18R

**TABLE 3.15 GROWTH IN CULTURE**

Control Pop Expansion	Low Population Expansion	Moderate Population Expansion	High Population Expansion
208F 1.53	M8 1.20 MT4 1.44 H16R 1.52 M7 1.54	MT9 1.67 H18 1.71 T3 1.77 H16 2.19 MT10 2.30 M1 2.89	T1 3.56 T2 3.67 H18R 3.71 MT7 4.06
Control Apoptotic Index	High Apoptotic Index	Moderate Apoptotic Index	Low Apoptotic Index
208F 2.62	M7 41.52 M8 15.44	T3 8.44 MT9 5.95 M1 4.57 MT4 4.10 MT10 2.30 T2 1.83 H18 1.56 H16 1.23 H16R 1.22	T1 0.77 H18R 0.74 MT7 0.62

**TABLE 3.16 GROWTH AS TUMOURS**

Control Size	Small Tumour Size	Moderate Tumour Size	Large Tumour Size
208F 0.29	M8 0.13 M1 0.37 H16 0.41 M7 0.51	H18R 0.92 H16R 0.99 MT9 1.02 H18 1.05 MT7 1.41 MT10 1.42	T1 2.20 T3 2.20 T2 2.24 MT4 4.65
Control Necrosis	Low Necrosis	Moderate Necrosis	High Necrosis
208F 0	M1 2 M8 5 MT4 9	M7 13 MT9 13 T3 14 MT10 17 H18 17	T2 22 H18R 22 H16 27 H16R 28 T1 32 MT7 40
Control Mitosis	Low Tumour Mitosis	Moderate Tumour Mitosis	High Tumour Mitosis
208F 1.03	M8 1.95 M1 3.4 T3 6.4 M7 9.6	MT4 11.7 MT9 12.2 H16 12.8 H16R 14.9 MT10 16.1 H18 16.7 H18R 17.0	T1 18.2 T2 18.2 MT7 26.1
Control Apoptosis	High Tumour Apoptosis	Moderate Tumour Apoptosis	Low Tumour Apoptosis
208F 2.8	MT4 40.3 M7 22.6 H16 20.3	M8 12.9 T2 11.6 H18 11.5 T3 11.1 MT10 10.9 M1 7.3	H16R 6.2 MT7 4.1 T1 3.9 MT9 3.6 H18R 3.1
Control M/A ratio	Low M/A ratio	Moderate M/A ratio	High M/A ratio
208F 0.44	M8 0.16 MT4 0.30 M7 0.50 M1 0.50	T3 0.70 H16 0.73 H18 1.50 MT10 1.60	T2 1.90 H16R 3.14 MT9 3.50 T1 5.30 H18R 5.77 MT7 6.80

Transfectants were categorised into mild, moderate or marked levels of expression of phenotypic properties (tables 3.14 to 3.16), and these groups were considered together (table 3.17). Grouping of cell lines involved some simplification of the data, in particular in the definition of boundaries between categories. Where possible, boundaries were positioned at naturally occurring gaps in the numerical distribution of the data. Despite this limitation, categorisation allowed simpler visualisation of the clustering of phenotypic properties amongst the 14 transfectants. Measures of proliferation in culture revealed very similar levels for all transfectants, and therefore these were not included in analysis of phenotypic clustering.

Morphological categorisation (table 3.14) indicated one group that contained the 3 myc transfectants M8, M7 and M1, as well as MT4 (derived from M1), and these often formed tight fascicles of cells which were poorly refractile and spindle-shaped to only a mild degree. The second group showed moderate degrees of refractility and spindle cell shape, and comprised H16, H18, T3, MT9, and MT10. The third group displayed marked morphological transformation, as judged by focus formation, spindle cell shape and refractility, and included the ras transfectants T1 and T2, the derivative of T1 (MT7), and the HPV and ras cotransfectants H16R and H18R. Thus, myc and ras tend to polarise at opposite ends of the



spectrum of morphological change.

The group of transfectants showing prominent fascicle formation, that included M7 and M8, and to a lesser extent M1 and MT4, often showed similar degrees of expression of other phenotypic properties (tables 3.14 - 3.17). These c-myc containing lines had low levels of net growth both in vitro (population expansion) and in vivo (tumour size). They showed high levels of apoptosis in culture (apoptotic index) and in tumours, relative to mitosis (M/A ratio). The effects of myc appeared to dominate in the serial transfectant MT4, derived from M1. These data indicate that c-myc induced slow net growth with high levels of tumour cell apoptosis.

The group of transfectants that displayed marked refractility and focus formation, including the T24-ras containing lines T1, T2 and MT7, and to a lesser extent H16R and H18R, also showed other similarities. They had high levels of population expansion and tumour size, but low apoptosis in culture and in tumours. T24-ras appeared to dominate in the MT7 serial transfectant, derived from T1. This pattern of clustering indicated that T24-ras stimulated fast net growth with suppression of tumour cell apoptosis.

The inclusion within this group of the combined HPV + T24-ras transfectants, H18R more so than H16R, perhaps reflects the dominance of the effects of ras over HPV genes. H16 and H18, which contained HPV genomes alone,

were mostly intermediate in phenotypic properties, although H16 showed a higher value for tumour apoptosis than H18. These findings suggested that HPV 16 may stimulate tumour cell apoptosis to a greater extent than HPV 18.

The serial transfectants containing both c-myc plus T24-ras showed patterns of apoptosis and net growth, in culture and in tumours, which broadly paralleled their cell of origin. MT4 (derived from the c-myc transfectant M1) showing a high level of apoptosis, and MT7 (derived from the T24-ras transfectant T1) showing a low level of apoptosis. The cotransfectants MT9 and MT10 showed intermediate values for apoptosis and net growth.

There is sufficient phenotypic clustering of the cell lines (table 3.17), with myc and ras often polarised at opposite ends of the spectra, to suggest that tumour behaviour may depend critically on expression levels of oncogenes. This would probably be best measured by enzyme-linked immunosorbent assay (ELISA) of the protein products in cells growing under different conditions: in culture or as tumours.

**TABLE 3.17 SUMMARY OF PHENOTYPIC PROPERTIES**

	+	++	+++
MORPHOLOGY	M8 M7 M1 MT4	H16 H18 T3 MT9 MT10	T1 T2 MT7 H16R H18R
POPULATION EXPANSION	M8 M7 MT4 H16R	H16 H18 M1 T3 MT9 MT10	T1 T2 MT7 H18R
APOPTOTIC INDEX	M8 M7	H16 H18 H16R M1 T2 T3 MT4 MT9 MT10	T1 MT7 H18R
TUMOUR SIZE	M8 M7 M1 H16	H18 H16R H18R MT7 MT9 MT10	T1 T2 T3 MT4
TUMOUR MITOSIS	M8 M7 M1 T3	H16 H18 H16R H18R MT4 MT9 MT10	T1 T2 MT7
TUMOUR APOPTOSIS	M7 H16 MT4	H18 M8 M1 T2 T3 MT10	T1 MT7 MT9 H16R H18R
TUMOUR M/A RATIO	M8 M7 M1 MT4	H16 H18 T3 MT10	T1 T2 MT7 MT9 H16R H18R

### 3.6.3 APOPTOSIS IN VITRO AND IN VIVO

Cellular bodies released into the culture medium by growing tumour cells were unequivocally shown to be apoptotic, both by morphological observation and demonstration of chromatin cleavage into oligonucleosomes.

Tumour cell apoptosis was demonstrated to be independent of cell proliferation both in vitro and in vivo. In culture, 2 out of three T24-ras transfectants (T1 and T2) had reduced apoptotic indices compared with controls, and two out of three c-myc transfectants (M7 and M8) showed considerably increased apoptosis. The rank order for the levels of apoptosis of these examples of single oncogene transfectants was identical in tumours. Scatter plot comparison of log apoptotic index in culture with log A/M measured in tumours, shows a strong correlation of  $r = 0.72$  (figure 3.25). Thus, apoptosis appears to be an intrinsic feature of cell lines, rather than being solely determined by the environment. This is consistent with genetic regulation of tumour cell apoptosis, perhaps via priming of cells for apoptosis (see chapter 4). However, environmental factors may have additional effects on the intrinsic rate of apoptosis, in particular by triggering the process by either specific or non-specific stimuli.

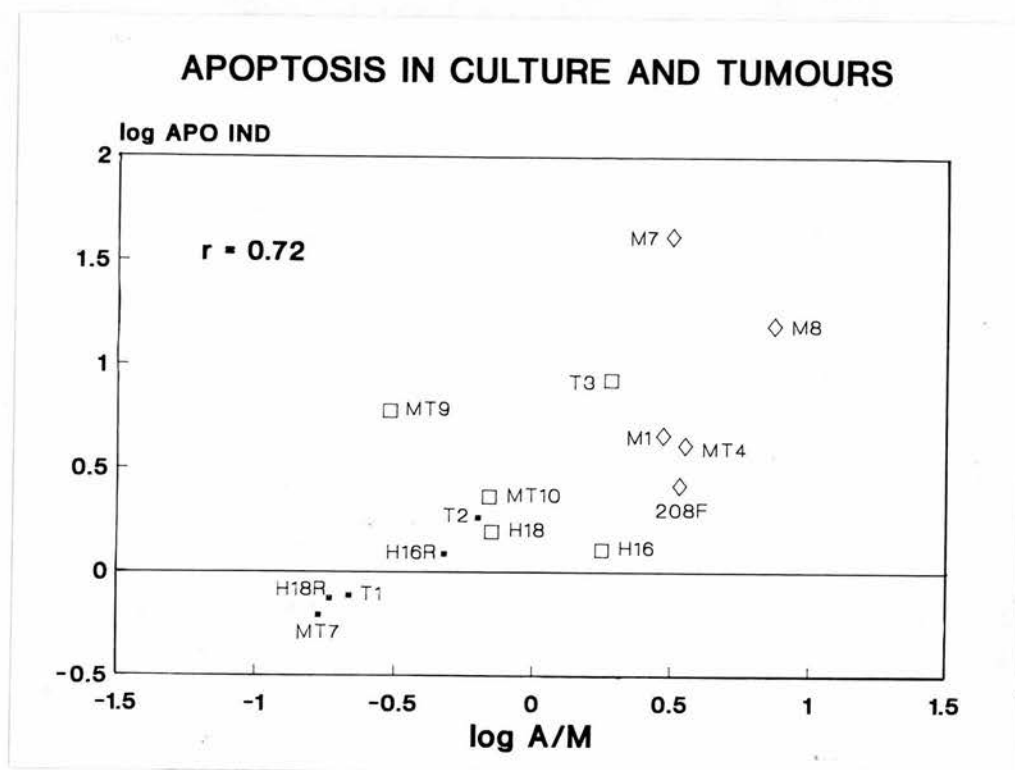


Figure 3.25

Scatter plot of log apoptotic index of transfectants growing in culture versus log A/M ratio of tumours formed in immune suppressed mice. Three sets of symbols are used that correspond with the families of transfectants with clustering of phenotypic properties, discussed in section 3.2. The parent control, 208F, is included in the *myc* transfectant family. There is a good correlation between these two measures of apoptosis (correlation coefficient  $r = 0.72$ ). This suggests that each cell line maintains an intrinsic rate of apoptosis either in culture or as a tumour.

#### 3.6.4 RELATIONSHIP OF POPULATION EXPANSION IN CULTURE WITH CELL BIRTH AND CELL DEATH

Analysis of growth parameters in culture revealed that proliferative indices of the 14 transfectants showed almost no significant differences. However, variations were observed in both the rates of cell population expansion and the rates of apoptosis, using dynamic measures of changes in cell or apoptotic body numbers over time. Population expansion measures and apoptotic indices showed a strong inverse correlation ( $r = -0.84$ ; figure 3.26) for the ten oncogene transfectants, excluding 208F. Similarly, the 4 cell lines containing high risk HPVs with or without T24-ras, showed a strong inverse correlation ( $r = -0.87$ ; figure 3.27). There was no other obvious means of exit from the proliferating pool (such as necrosis or differentiation). Thus, the rate of apoptosis appears to be the major regulator of the rate of population expansion for these cell lines growing in culture.

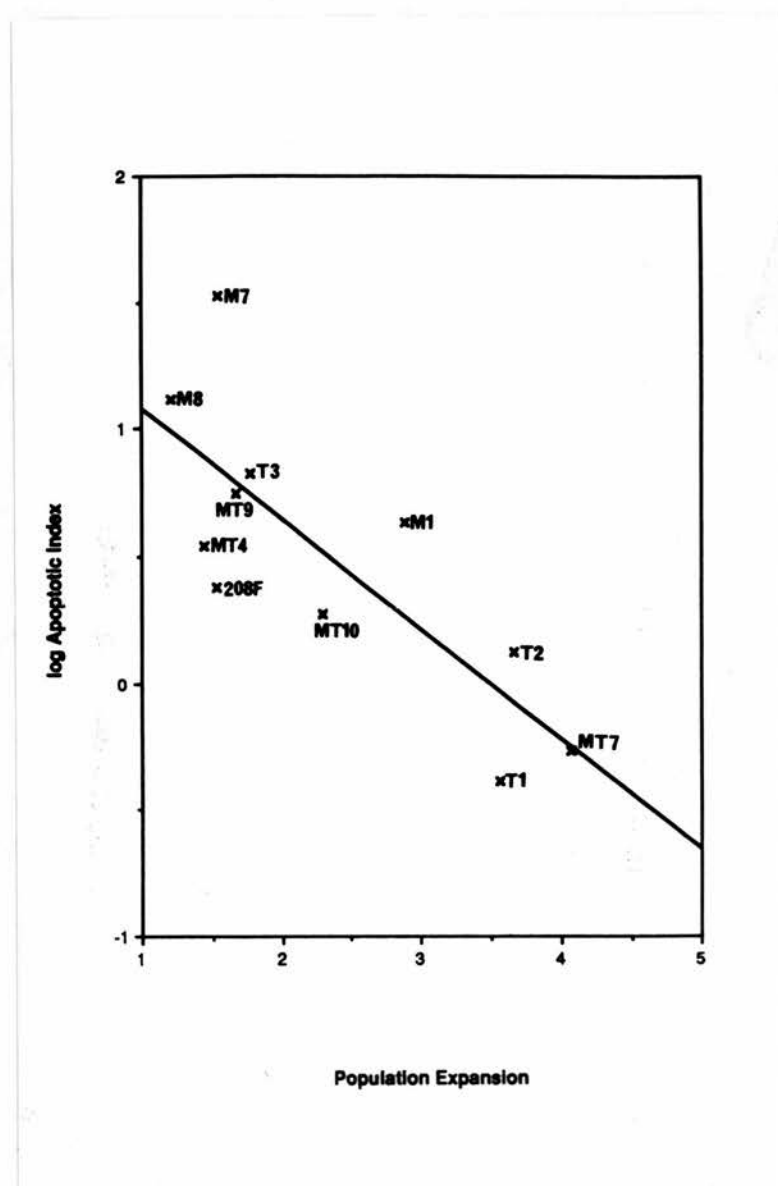


Figure 3.26

Graph of log apoptotic index versus population expansion rate for the oncogene transfectants growing in culture. The scatter plot shows a strong inverse correlation (correlation coefficient  $r = -0.84$ ) for the 10 oncogene transfectants. Two of the 3 single myc transfectants, M7 and M8, are seen at the top of the graph with high apoptosis but low population expansion, whereas 2 of the 3 single ras transfectants, T1 and T2, and the T1 derivative, MT7, are seen at the bottom of the graph with high population expansion and low apoptosis.

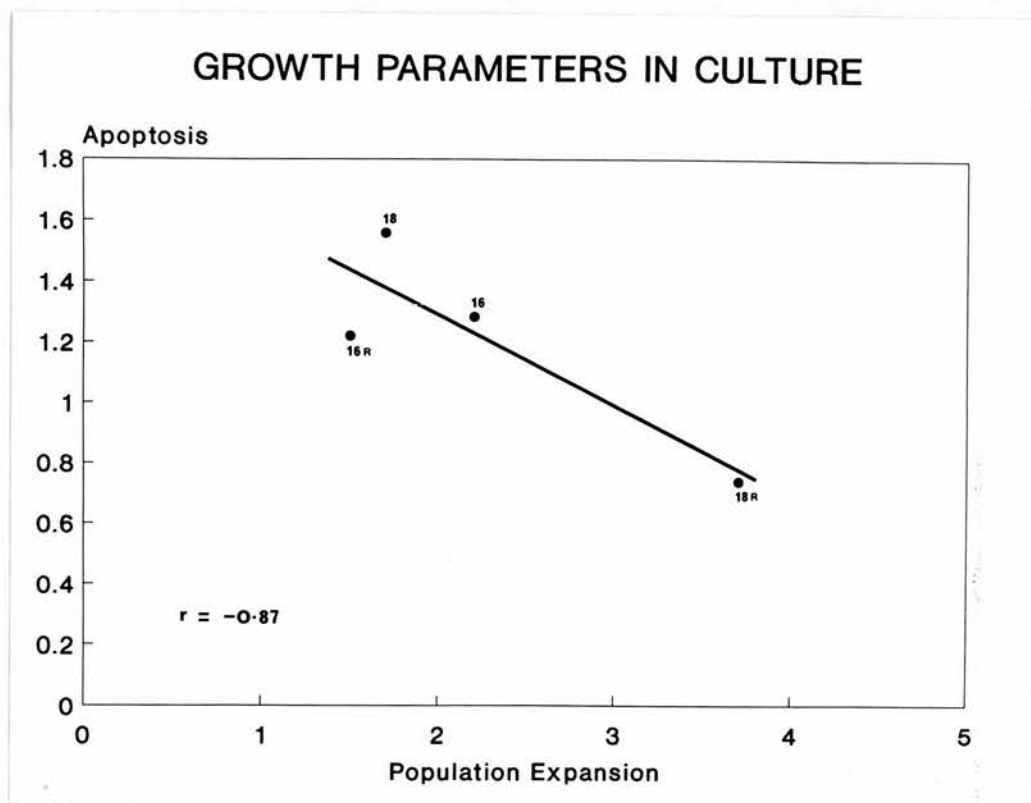


Figure 3.27

Graph of apoptotic index versus population expansion rate for the 4 HPV transfectants growing in culture. The scatter plot shows a strong inverse of correlation (correlation coefficient  $r = -0.87$ ). The 3 cell lines H16, H16R and H18 are clustered together with moderate to high apoptosis and low population expansion, whereas H18R shows low apoptosis and high population expansion.



### 3.6.5 REGULATION OF APOPTOSIS BY ONCOGENES AND HPV

The effects of myc and ras on apoptosis were shown clearly in 2 out of 3 examples for each set of single oncogene transfectants. C-myc was associated with stimulation of apoptosis, and T24-ras with suppression of apoptosis. The 2 exceptions were the T3 and M1 transfectants. Preliminary immunocytochemical experiments to determine the levels of expression of the p21ras protein have shown that T1, and to a lesser extent T2, contained high levels of ras product, whereas T3 expressed the ras oncogene at lower levels, and these data showed a strong inverse correlation with measures of apoptosis in culture (N. Toft, personal communication). Southern blotting showed a similar rank order of cell lines in terms of approximate ras gene copy number indicated by strength of hybridisation signal (figure 3.8A). This suggests that the high rates of apoptosis demonstrated by T3 may have occurred as a result of expression of ras protein at a level too low to effectively suppress apoptosis.

Tumours generated by the M1 transfectant showed higher primary tumour take rates, and lower tumour apoptotic counts in this study compared to those previously recorded (Wyllie et al, 1987). One explanation may be that this cell line has undergone selection for growth advantage over the four to five year period between its construction by Spandidos and Wilkie (1984), and

experimental analysis in this study. Given the similarity of measures of cell proliferation in culture shown by the different cell lines studied here, such evolution of a faster growing M1 subclone may have occurred by reduction of apoptosis.

A pattern of differential regulation of apoptosis was also observed in the behaviour of transfectants containing high risk HPV genomes. Those with HPV alone had moderate to high levels of apoptosis in vivo and in vitro. During tumour growth HPV 16 was associated with higher apoptosis and lower M/A ratios than HPV 18. Cotransfectants containing both HPV and T24-ras were associated with lower apoptosis in vivo compared to those containing HPV alone, presumably reflecting the strong influence of ras in suppression of apoptosis. This was particularly notable for HPV 18 plus ras, which also displayed decreased apoptosis in vitro. Of the two high risk HPV types, tumours containing HPV 18 showed less apoptosis than those containing HPV 16, with or without activated ras. In summary, there is further evidence that activated ras appeared to suppress apoptosis, and both high risk HPVs appeared to stimulate tumour cell apoptosis, HPV 16 more so than HPV 18, though to a lesser extent than c-myc.

### 3.6.6 RELATIONSHIP OF NET TUMOUR GROWTH AND CELL TURNOVER

There was an interesting relationship between tumour size and cell turnover, calculated as a single index using the mitosis/apoptosis ratio (figures 3.22 and 3.24). High M/A ratios were observed in large and moderately-sized tumours formed by the T24-ras-containing transfectants T1, T2, MT7, H16R and H18R, whereas low M/A ratios were found in small tumours generated by the 3 c-myc transfectants M8, M7 and M1. However, a poor correlation of  $r = -0.18$  for these two parameters is shown in the scatter plot of tumour size versus log A/M for all cell lines (figure 3.28). The level of correlation increases to  $r = -0.63$  if the outlier MT4 is excluded. Scatter plot comparison of the population expansion data with tumour sizes for all cell lines (figure 3.29), also shows a poor correlation of  $r = 0.20$  (increases to  $r = 0.66$  if MT4 is omitted). Large tumour size appears to relate more simply to presence of T24-ras, as shown by the 5 cell lines (MT7, T1, T2, T3 and MT4) which generate tumours bigger than 2.0 on the scale used.

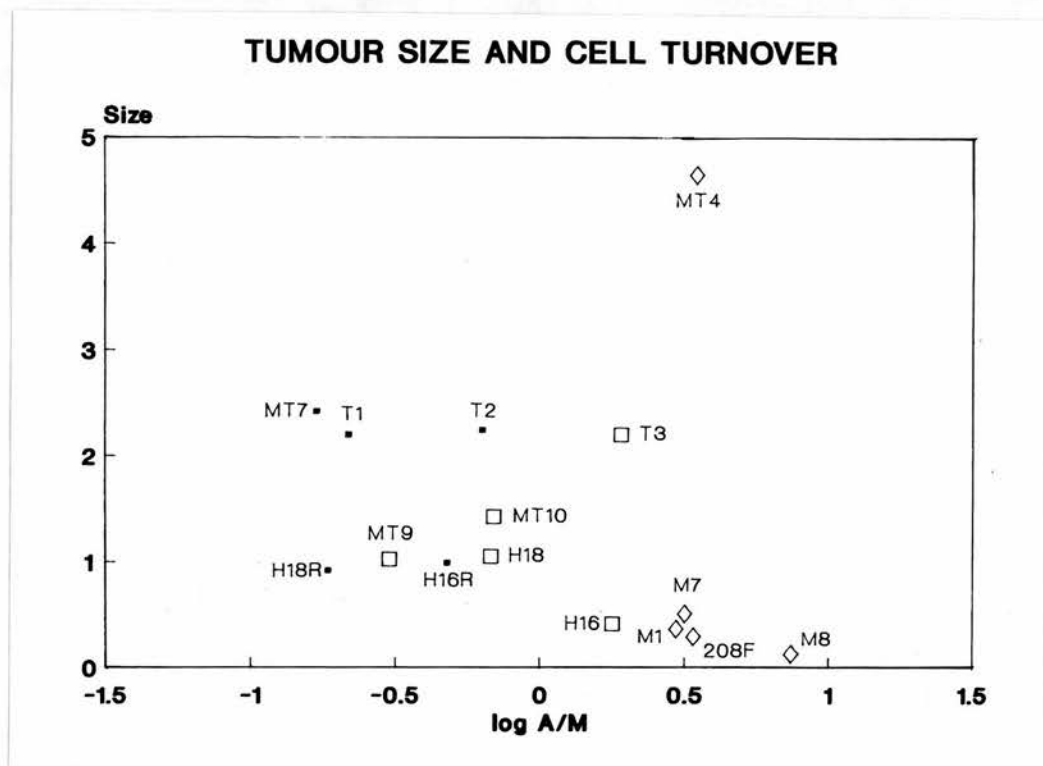


Figure 3.28

Scatter plot of tumour size versus log A/M ratio for all cell lines growing as tumours in immune-suppressed mice. There appears to be a trend towards a loose inverse correlation between size and cell turnover, with the exception of MT4 which shows large size and high log A/M ratios.

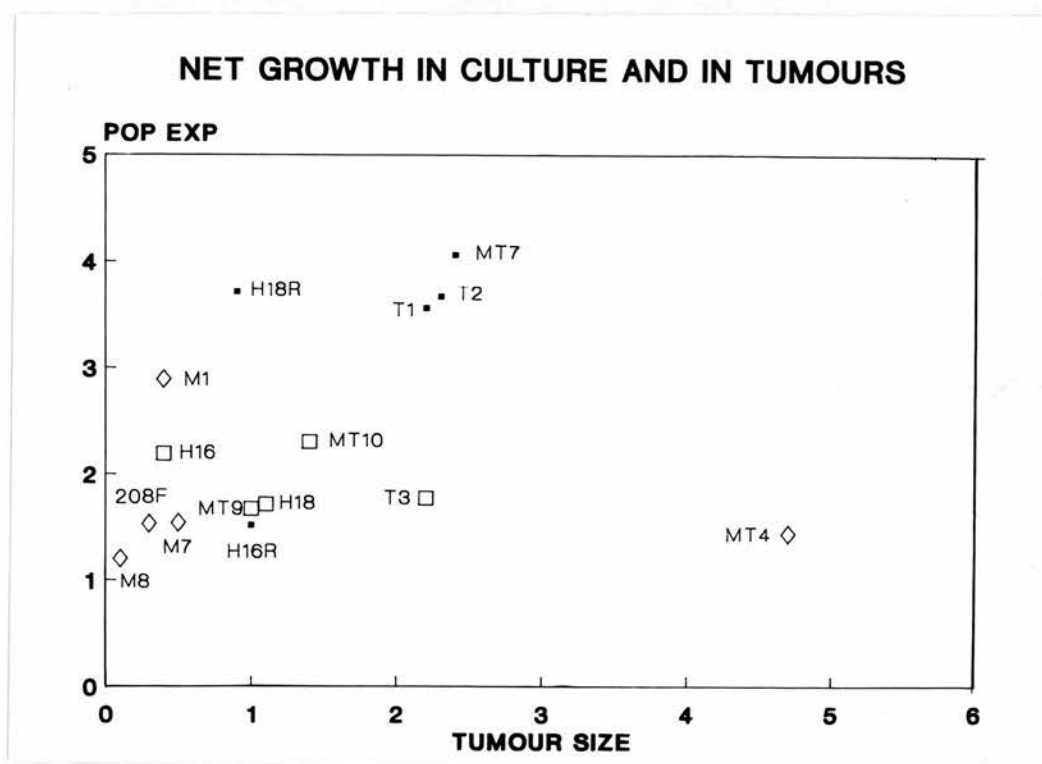


Figure 3.29

Scatter plot of population expansion rate for transfectants growing in culture versus tumour size in immune suppressed mice. There is a trend towards a loose correlation between these 2 measures of overall growth, with the exception of MT4 which shows a very high tumour size but only a low population expansion rate.

In tumours, both mitosis and necrosis showed variability between transfectants, and hence affected net tumour growth. The measure of cell turnover (A/M ratio) reflects levels of apoptosis relative to those of mitosis and cancels out any bias due to differences in cell size and density. Thus, differing levels of necrosis may account for the poor correlation of tumour size with cell turnover as measured by the A/M ratio. This indicates a more complex relationship of these parameters affecting net growth in vivo, compared with that in culture, due to other, possibly limiting, factors such as angiogenesis (Denekamp and Hobson, 1982; Tozer et al, 1990).

### 3.6.7 TUMOUR NECROSIS

High levels of necrosis were seen mostly in transfectants containing the T24-ras oncogene that formed moderate or large tumours (T1, T2, MT7, H16R and H18R). Necrosis appeared to be of minor importance in transfectants containing the c-myc oncogene (M8, M7, M1 and its derivative MT4).

Scatter plot comparison of tumour necrosis with cell turnover ( $\log A/M$ ) for all cell lines (figure 3.30) shows a strong inverse correlation of  $r = -0.76$ . This may reflect a relationship of necrosis to mitosis that relates to the traditional view of tumour necrosis occurring as a result of rapid tumour growth outstripping the provision of blood supply (Thomlinson and Gray, 1955; Jones and Caplejohn, 1983; Tozer et al, 1990). Furthermore, the reduced capacity to undergo apoptosis of ras transfectants may necessitate necrosis as the only mode of cell deletion available for cells subjected to an insufficient blood supply.

The overall tumour size, which reflects the balance of mitosis, apoptosis and necrosis, correlates poorly with tumour necrosis (figure 3.31) with a correlation coefficient of  $r = 0.26$  (increases to  $r = 0.65$  if MT4 is omitted). The MT4 transfectant (derived from M1) differs from the others in several respects, in that it showed very high apoptosis, but generated the largest tumours.

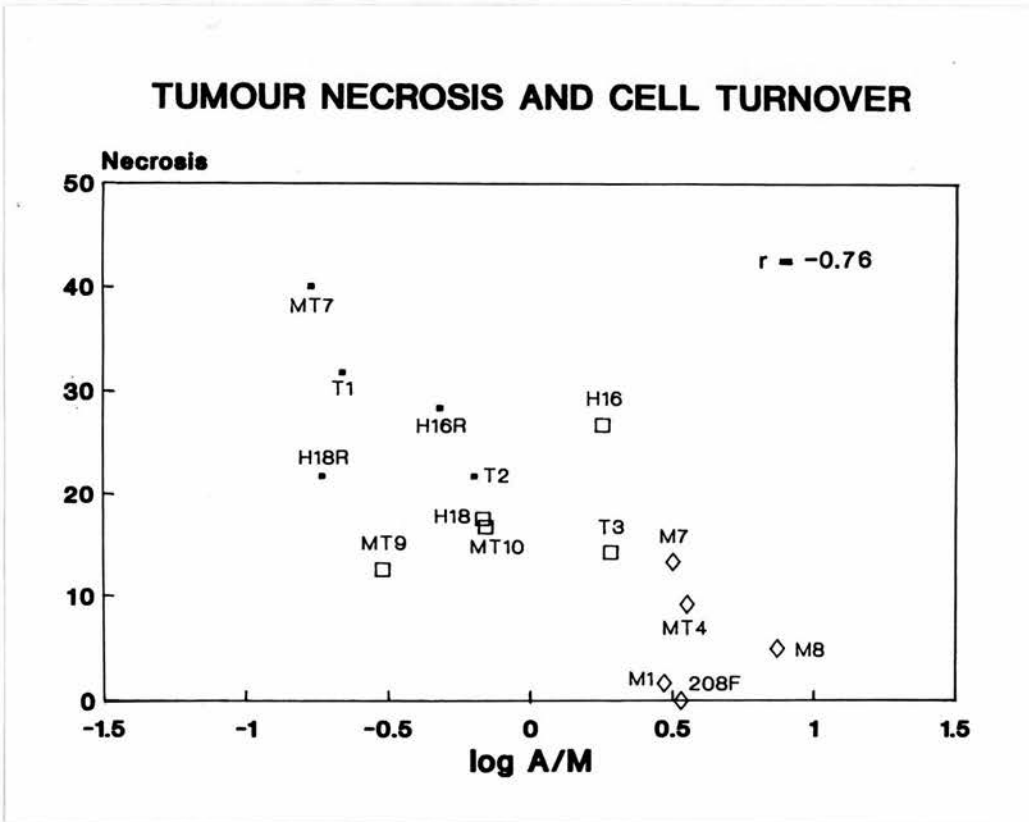


Figure 3.30

Scatter plot of tumour necrosis versus log A/M ratio of transfectants growing as tumours in immune suppressed mice. There is a good inverse correlation between necrotic cell death and this indirect measure of apoptotic cell death relative to mitosis (correlation coefficient  $r = -0.76$ ).



The reasons for this are not apparent, and MT4 makes an interesting subject for further investigation. One possibility is that some combination of moderate mitosis but low necrosis, resulted in fast net growth despite high apoptosis.

Axes may be positioned, somewhat arbitrarily, on the scatter plot of size versus necrosis (figure 3.31) that divide the transfectants into 4 groups that relate necrosis to the transfected gene and to morphology. The 3 c-myc transfectants M1, M7 and M8, together with the parent 208F (and to a lesser extent MT9) generated small tumours with low necrosis. The 4 HPV containing lines formed small tumours, but with high necrosis. T1, T2 and MT7, which contained T24-ras (and MT10 to a lesser extent), produced large tumours with much necrosis. Only MT4 and T3, which are exceptional in other regards, formed different sized tumours with low necrosis.

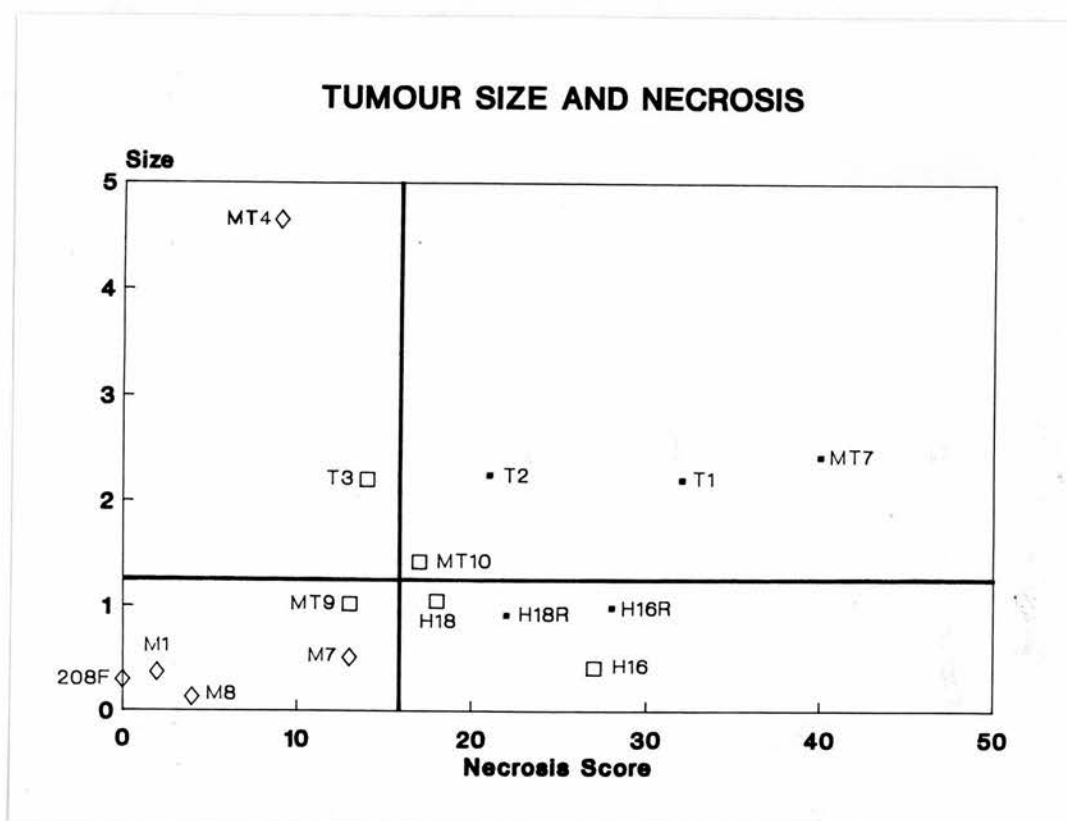


Figure 3.31

Scatter plot of tumour size versus tumour necrosis for transfectants growing in immune suppressed mice. Arbitrary axes have been superimposed on the scatter plot, which divide the transfectants into 4 major groups. Myc transfectants and 208F form small tumours with low necrosis. The 4 HPV transfectants form tumours of low to moderate size with high necrosis. MT4 and to a lesser extent T3 form large tumours with little necrosis. The ras transfectants T1 and T2, together with MT7 form large tumours with large amounts of necrosis. MT9 and MT10 are intermediate in both tumour size and necrosis.

### 3.7 CONCLUSIONS

1. Fibroblast tumour cell death in culture is by apoptosis, characterised ultrastructurally and by chromatin cleavage.

2. The apoptotic rate of each tumour cell line appears to be intrinsically determined. There is a strong correlation between apoptosis in vitro and in vivo.

3. Apoptosis is a key determinant of net growth in vitro, as it shows a strong inverse correlation with the rate of population expansion. Variability in tumour mitosis and necrosis ensure a more complex relationship of apoptosis with tumour size.

4. C-myc stimulates tumour cell apoptosis, as do HPV types 16 and 18, although to a lesser extent. In contrast, activated c-Ha-ras1 suppresses apoptosis, either alone or in combination with other genes.

#### 4. **FINAL DISCUSSION**

4.1 Transition from CIN to Cervical Cancer

4.2 High Risk HPVs Influence Apoptosis

4.3 Regulation of Tumour Cell Apoptosis by Ras, Myc and HPV E6 and E7 Genes

4.4 Two Classes of Event are Required for Apoptosis

4.5 Myc and Ras Control Cell Fate

This chapter will attempt to synthesise clinico-pathological data about the influence of high risk HPVs on neoplastic progression from CIN to cervical cancer described in chapter 2, with the effects of HPVs on cell proliferation and apoptosis set out in chapter 3. This leads on to a discussion of the regulation of apoptosis by ras and myc, including possible commonality of mechanism of HPV E7 and myc, and suppression or activation of priming and triggering for apoptosis. The regulation of apoptosis is finally discussed within a wider view of the control of cell fate, in terms of transition between stable states of growth arrest, rapid turnover, and population expansion.

#### 4.1 TRANSITION FROM CIN TO CERVICAL CANCER

The transition from precursor to malignancy and the development of metastatic potential represent the two key neoplastic changes that threaten the life of the patient. In the cervix, the immediate precursors are high grade CIN lesions (CIN 2/3), shown earlier in this thesis to contain high risk HPV types 16 and 18 in 60% of cases. Cancers contain these HPV types in 70% - 80% of cases. These data indicate a greater tendency for HPV-associated CIN lesions to progress to malignancy compared with non-HPV containing precursor lesions. This suggests that HPVs influence the transition.

An earlier discussion presented the view that a more rapid transition from CIN to cancer appears to be associated with HPV 18 relative to HPV 16. This was based on the evidence of two - five fold greater CANCER/CIN prevalence ratios for HPV 18 compared with HPV 16, depending on the groups of CIN or carcinoma lesions compared. Many possible explanations may be suggested for differences in relative aggression between these two viral types (see below).

The rate of production of new CIN cells is one factor that is likely to directly affect the probability of further genetic change required for malignancy, such as integration of the HPV genome, activation of cellular oncogenes or loss of oncosuppressor genes. This thesis has shown that the rate of generation of cells results

from the balance of cell gain and cell loss. Different levels of tumour cell apoptosis were observed for HPV 16 and 18.

#### 4.2 HIGH RISK HPVs INFLUENCE APOPTOSIS

The two high risk HPV types were associated with moderate to high levels of tumour cell apoptosis in vivo. HPV 18 was associated with lower levels of tumour apoptosis and A/M ratio than HPV 16. In combination with ras, the HPV 18 transfectant, H18R, showed faster population expansion in culture than H16R.

Explanations for the differences between these two high risk HPV types may be sought at the level of molecular interactions of their protein products. The E7 protein of HPV 18 binds Rb with a higher affinity than HPV 16 E7 (Phelps et al, 1988; Munger et al, 1989b), and also has a faster rate of casein kinase II phosphorylation than HPV 16 E7, both of which are more rapid than the rates for low risk HPV E7 proteins (Barbosa et al, 1990).

The efficiency of transformation of 208F shown in this thesis is greater for HPV 18 than for HPV 16, and this is consistent with a five-fold more efficient rate of immortalisation of human cervical keratinocytes by HPV 18 compared with HPV 16 (Barbosa and Schlegel, 1989).

A further reason for some of these differences is that compared with HPV 16, HPV 18 has a more efficient

transcriptional regulatory region producing higher levels of expression of the E6 and E7 genes. This was shown to be a major determinant of their different immortalising activities (Romanczuk et al, 1991).

Phenotypic analysis of authentic human cervical cancers containing these viral genomes shows greater aggression for HPV 18 in terms of neoplastic progression from CIN to cancer, cancer cell differentiation, and higher tumour grade (Wilczynski et al, 1988; Barnes et al, 1988; Kurman et al, 1988; chapter 2 of this thesis).

In theory, it is possible to link reduced apoptosis with a more rapid transition from CIN to cancer. If the lower apoptosis and faster growth rate associated with HPV 18 in this fibroblast system, also occurred in cervical keratinocytes in vivo, this would result in more rapid production of CIN cells, which is likely to increase the probability of further genetic changes required for transition to malignancy. Furthermore, selection pressures may be different, in that reduced apoptosis may allow survival of genetically altered variants which might otherwise die in a population of tumour cells more prone to apoptosis. This raises the question of how viral and cellular oncogenes regulate tumour cell apoptosis.

#### 4.3 REGULATION OF TUMOUR CELL APOPTOSIS BY RAS, MYC AND HPV E6 AND E7 GENES

##### A. RAS

Suppression of tumour cell apoptosis by activated ras has been shown in this thesis to produce faster population expansion in culture and contribute towards larger tumour size. Increased production of cells is likely to influence the degree of aggression of neoplasms. A key difference between ras and myc tumour behaviour investigated by Wyllie et al (1987), was the aggressiveness of the tumours that they formed: the T24-ras expressing cells produced a higher primary tumour take rate, a higher proportion of test mice with metastasis at 14 days, and a higher cytological grade of fibrosarcoma, compared with c-myc expressing cells.

Carcinogen-induced mutation of ras followed by loss of the normal ras allele is associated with progression of established squamous carcinomas to more aggressive spindle cell cancers (Brown et al, 1986; Buchmann et al, 1991). Activation of ras also occurs in premalignant lesions, such as colorectal adenomas (Williams et al, 1990), and atypical hyperplasia of breast (Going et al, 1992). Faster population expansion of precursors in these circumstances may be important in increasing the risk of malignant transformation.

Ras-mediated inhibition of apoptosis is further supported by two other findings. Ki-ras expression decreases in



association with the onset of apoptosis in rat chloroleukaemic cells (Rytomaa and Servomaa, 1987). The ras oncogene has also been implicated in suppression of cell death of mouse mast cells. An immortalised interleukin-3 dependent mouse mast cell line (BB-3c) was transfected with the human activated Ha-ras oncogene under the transcriptional control of the mouse mammary tumour virus long terminal repeat that contains a steroid-responsive enhancer element (Andrejauskas and Moroni, 1989). Steroid treatment produced expression of ras p21 protein which was associated with progressive growth of the transfectants in the absence of exogenous interleukin-3. On withdrawal of steroid, p21 ras protein expression decreased and this was associated with rapid cell death. Expression of the Ha-ras oncogene induced the cell line to produce autocrine interleukin-3 and granulocyte macrophage colony stimulating factor, suggesting a complex mechanism of growth control implicating ras stimulation of autocrine growth factor secretion and suppression of cell death. Interleukin-3 has also been shown to rescue other leukaemic cell lines from apoptosis (Lotem et al, 1991).

The mechanism by which ras may inhibit apoptosis is unknown, but one possibility is the prevention or reversal of the priming stage for apoptosis (see below), which may be an essential step prior to triggering of apoptosis by specific or non-specific stimuli.

The ras product is not the only oncoprotein implicated in suppression of apoptosis. The bcl-2 gene product has also been demonstrated to inhibit apoptosis. It was first identified through gene mapping of the t(14;18) chromosomal translocation of human follicular B-cell lymphoma, which juxtaposes the bcl-2 gene on chromosome 18 with the immunoglobulin heavy chain locus on chromosome 14. The bcl-2-immunoglobulin fusion gene is markedly deregulated resulting in inappropriately elevated levels of bcl-2 RNA and protein (Cleary et al, 1986). Follicular lymphoma is typically an indolent disease comprising small resting B-cells, which frequently develops into a high grade lymphoma.

Bcl-2 encodes proteins of 22-26 kDa (Haldar et al, 1989), that have been localised to the inner mitochondrial membrane (Hockenbery et al, 1990). Overexpression of bcl-2 has been shown to block apoptotic cell death of a pro-B lymphocyte cell line (Hockenbery et al, 1990). Moreover, deregulated bcl-2 extends the survival of certain haematopoietic cell lines following growth factor deprivation (Vaux et al, 1988; Nunez et al, 1990). Transgenic mice bearing a bcl-2-immunoglobulin fusion minigene showed polyclonal expansion of resting IgM-IgD B cells which display prolonged cell survival but no increase in cell proliferation (McDonnell et al, 1989), and following a long latency there was progression from polyclonal to monoclonal disease, in which half of the tumours were immunoblastic high grade lymphomas with

a rearranged c-myc gene, suggesting that prolonged B cell life increased tumour incidence (McDonnell and Korsmeyer, 1991).

The bcl-2 protein was expressed in variety of cell lineages in transgenic mice, including but not exclusive to those with a high incidence of cancer in man, such as skin, colon, breast, prostate and pancreas, raising the possibility that bcl-2-induced extended survival of such cell types may contribute to their predisposition to malignancy (Hockenber y et al, 1991).

The effects on tumour cell apoptosis have also been studied in Epstein-Barr virus containing tumours. EBV-positive BL cell clones expressing only one virus latent protein (EBNA-1) have been shown to be extremely sensitive to apoptosis, whereas isogenic clones expressing all 8 EBV latent proteins were considerably more resistant to the induction of apoptosis, suggesting enhancement of B cell survival by EBV-induced suppression of apoptosis, and this may contribute to either B cell infection or neoplasia (Gregory et al, 1991). The latent membrane protein LMP-1 mediates this suppression of apoptosis in transfected human B cells, and is associated with upregulation of bcl-2 (Henderson et al, 1991).

## B. MYC

In marked contrast to rescue from apoptosis induced by ras or bcl-2 genes, c-myc increased tumour cell apoptosis in vitro and in vivo. This correlated with low population expansion rates and small tumour sizes. Experimental modulation of c-myc activity has also been used to demonstrate that myc stimulates apoptosis in Rat-1 fibroblasts transfected with a construct encoding a myc-oestrogen receptor (MYC-ER) fusion protein which only shows myc activity in the presence of oestrogen (Eilers et al, 1989; Evan et al, 1992). This experimental system showed that the degree of apoptosis was related to the levels of myc protein in cells, and that constitutive myc expression induced apoptosis in cells growth arrested by various means including serum deprivation. The regions of the myc protein required for apoptosis were studied using a range of mutants, and these were the same as those required for cotransformation, autosuppression and inhibition of differentiation (Evan et al, 1992).

Increased expression of c-myc has been found during apoptosis of rat prostate epithelium following castration (Buttayan et al, 1988). Thus, myc is associated with induction of apoptosis in mesenchymal and epithelial cell types.

### C. HPV E6 AND E7 GENES

The c-myc oncogene is associated with high levels of apoptosis, and high risk HPV genomes are associated with moderate levels. It is possible that this similarity of effect may be due to common mechanisms of action. HPV E7 transforming gene products (of high risk types 16 and 18) have been demonstrated to bind the retinoblastoma protein p105 (Phelps et al, 1988; Munger et al, 1989b and 1991). Functional inactivation of retinoblastoma gene products, by binding with viral transforming proteins, has been shown to lead to release of TGF-beta1 inhibition of c-myc transcriptional activity within keratinocytes (Pietenpol et al, 1990).

There is evidence of direct binding in vitro of c-myc protein to Rb protein, through a common Rb "pocket". This "pocket" also mediates Rb binding to the cellular transcription factor E2F, several other cellular nuclear proteins, and the DNA tumour virus oncoproteins SVLT, AdE1A, HPV 16 E7 and HPV 18 E7 (Wagner and Green, 1991). The N terminal domain of c-myc protein contains the binding site for Rb and competes directly with HPV E7 (Rustgi et al, 1991), whereas the DNA binding and protein dimerisation motifs are situated in the C terminus of c-myc (Cole, 1991). Furthermore, the c-myc promotor sequence contains many protein binding motifs including E2F-binding sites (Chittenden et al, 1991), and this promotor site is involved in Rb-mediated repression of c-

myc (Moses et al, 1990). The retinoblastoma protein can also repress c-fos expression by an effect on its 5' cis-acting promotor element ("Rb control element" or RCE, which contains sequence similarities to the E2F binding site) (Robbins et al, 1990).

Thus, HPV 16 and 18 E7 proteins inactivate Rb protein and may release repression of c-fos as well as c-myc transcription, and release c-myc protein from Rb-myc complexes. Both myc and fos are associated with stimulation of apoptosis as well as proliferation. C-fos and c-myc (along with hsp 70) have increased expression during apoptosis of rat prostate epithelium (Buttayan et al, 1988). It would be possible in future to investigate the levels of expression of myc and fos proteins in the HPV transfected cell lines.

Both c-fos and c-myc oncoproteins bind DNA and have been implicated in regulating gene expression, but there are few examples of direct effects on specific genes, particularly for c-myc. C-myc forms dimers with max, which bind the sequence CACGTG present in the plasminogen activator inhibitor-1 promotor that is deregulated in myc-immortalised fibroblasts (Cole, 1991). However, the region of the c-myc protein involved in binding to the Rb "pocket" is also a weak transcriptional activation domain and contributes to transformation (Cole, 1991).

The effects of the p53 tumour suppressor gene on tumour cell apoptosis have been investigated using a temperature

sensitive p53 mutant introduced into a murine myeloid leukaemic cell line. The Val-135 mutant behaves like other oncogenic p53 mutants at 37.5°C, but like wild-type p53 at 32.5°C. No effects on myeloid differentiation were observed, but wild-type p53 activity resulted in induction of apoptosis, not found with mutant p53 activity (Yonish-Rouach et al, 1991). The possibility that wild-type p53 has similar effects on other cell types has still to be studied. The actions of high risk HPV 16 and 18 transforming E6 proteins include binding cellular p53 protein (Werness et al, 1990), and producing increased p53 protein degradation (Scheffner et al, 1991). If wild-type p53 induces apoptosis in keratinocytes and fibroblasts, then E6 would be expected to counteract this, producing relative downregulation of apoptosis.

HPV E7-mediated inactivation of Rb may induce transactivation of both myc and fos, and HPV E6 produces degradation of wild-type p53. This combination of activities is likely to have pleiotropic effects. These may include effects on differentiation, proliferation and apoptosis, although this is likely to depend on other intracellular signalling activities, such as the presence or absence of ras activation. Inactivation of Rb may also release other cellular factors from complexes with Rb, such as E2F, which subsequently alter gene expression relevant to controlling cell turnover. The net effects on apoptosis are likely to result from the summation of the



predicted activities of E7 (stimulation of apoptosis via Rb effects on myc and fos) and E6 (suppression of apoptosis via p53), and this will be affected by the comparative levels of expression of E7 and E6, and their relative efficiencies in terms of protein function and stability, both of which may differ between HPV types. Overall, the moderate levels of tumour cell apoptosis observed in this study are consistent with a combination of positive and negative influences on tumour cell apoptosis.

HPV genomes may also exert an influence on tumour cell apoptosis by mechanisms independent of E7 and E6 effects. Other HPV genes, present in the transfected plasmids, may have effects that can not be excluded by these experiments: HPV E2 and E1-related gene products have also been demonstrated to bind DNA and influence viral gene expression, although there is little information available about their effects upon cellular genes. BPV1 also has transforming properties, but E5 functions in HPV 16 and 18 are unclear. Interestingly, few differences have been shown between the E1, E2 and E5 protein functions of low and high risk HPV types. These sequences can be deleted during viral genome integration, which is a key early event in cervical neoplasia. Thus, their effects on apoptosis, if any, in human cervical cancers may not be as significant as those of E6 and E7.



The dual binding of Rb and p53 is a common mechanism for three classes of DNA tumour viruses: SV40, adenovirus, and high risk HPVs. The E1a protein of adenovirus binds Rb and has been reported to have a "cytotoxic" effect on cells of unexplained mechanism (Whyte et al, 1988). Apoptosis was not investigated in this report. This commonality of viral protein function emphasizes the importance of the cellular targets, and suggests that there is a common pathway for transformation of a variety of cell types from several species. This provides some support for extrapolation of data from rat fibroblasts to human cervical cells.

#### 4.4 TWO CLASSES OF EVENT ARE REQUIRED FOR APOPTOSIS

Apoptosis depends upon the availability of certain key proteins. These include the calcium-magnesium sensitive endonuclease (Wyllie, 1980; Arends et al, 1990), and the glutamyl transferases (Fesus et al, 1989). It is probable that there are several more. Neither the endonuclease nor the transglutaminases are normally present in every cell in a tissue; they accumulate before apoptosis takes place. Their coordinate expression is presumably regulated by specific controller genes. In the paragraphs to follow, a model is proposed, in which this coordinate expression is referred to as **priming** for apoptosis (figure 4.1).

Some tissues include a high proportion of primed cells (eg thymus cortex) (Van Haelst, 1967a and 1967b), but in most (eg liver) (Zajicek et al, 1985) they normally represent a small minority. Only within the primed subpopulation can apoptosis occur. In these, the initiation of apoptosis is the result of a distinct set of events, referred to as **triggering**. Triggering mechanisms include the controlled influx of calcium into the cell, and cause activation of the endonuclease, transglutaminases, and other putative effectors of apoptosis. It is probable that triggered cells proceed inevitably into apoptosis, but priming may be reversible.

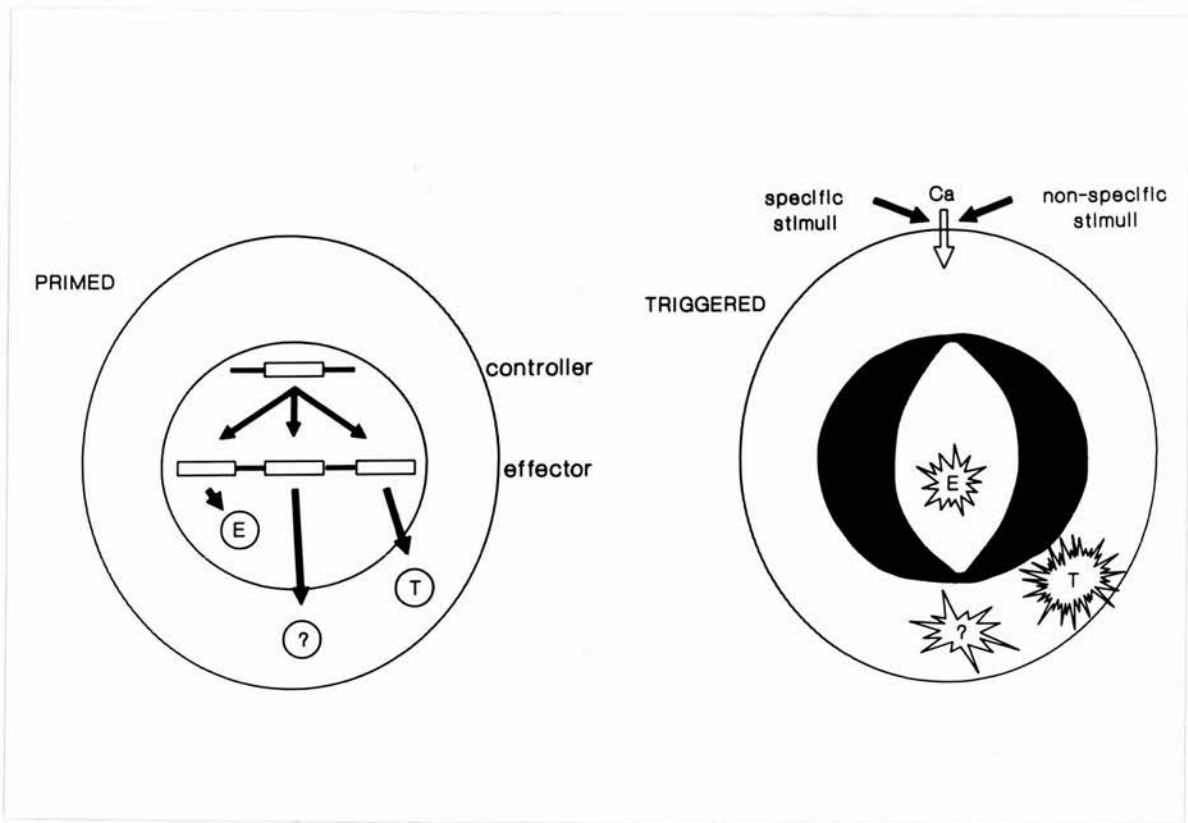


Figure 4.1

Two stage model for induction and activation of the effectors of apoptosis. Cells become primed for apoptosis, presumably in response to cell type-specific signals, by expression of inactive precursor effector proteins, such as the endonuclease (E), transglutaminases (T), and other putative effectors (?). Effector expression may be coordinately regulated by specific controller genes. Apoptosis is initiated in primed cells, through activation of effector molecules, by triggering mechanisms. In thymocytes, these include the controlled influx of calcium, without activation of protein kinase C, and occur in response either to specific stimuli, such as glucocorticoid or CD3 ligands, or to non-specific stimuli, such as calcium ionophore, TCD-dioxin or low to moderate doses of irradiation. Both endonuclease and transglutaminase require calcium ions for activation.

One of the actions of the activated ras oncogene, in this scenario, may be to prevent or reverse the priming of cells. Whereas, c-myc and high risk HPV E7 genes (possibly via c-myc) may stimulate both priming, perhaps by activating the putative controller genes.

This is supported by the presence of calcium-magnesium sensitive endonuclease activity in viable c-myc transfectants M1, M7 and M8, but not in the parent 208F, or T24-ras transfectant T1 cells. The serum sensitivity of expression of the endonuclease in the transfectants T2 and T3 may reflect the levels of ras protein within the cells following growth factor withdrawal. The level of p21ras protein has been shown to decrease moderately in T1 cells, and markedly in T2 and T3 cells (to unmeasurable levels using a crude serial dilution immunocytochemical method) when cultured in conditions of serum deprivation, and this correlated inversely with an increase in apoptosis (N. Toft, personal communication).

One of the interesting implications of this distinction between priming and triggering, is that triggering stimuli need not be as specific as those involved in priming. Apoptosis may be triggered by activation of specific surface receptors such as the T cell receptor (see below), APO-1 or the very similar fas molecule (Trauth et al, 1989; Itoh et al, 1991), and also by mild cellular injury capable of generating a temporary influx of calcium ions. This could constitute a triggering

stimulus to a primed cell, although it would have no such effect on an unprimed one.

Two predictions follow: primed cells should be vulnerable to apoptosis in response to a wide variety of minor injury stimuli, and apoptosis of this sort should occur exclusively in those regions of tissues in which primed cells lie. In fact, there is evidence supporting both these predictions. Thymocytes undergo apoptosis in response to specific stimuli as diverse as glucocorticoid or CD3 ligands, and to non-specific stimuli such as low to moderate doses of ionising radiation, TCD-dioxin or calcium ionophore (Van Haelst, 1967b; Umansky et al, 1981; Wyllie et al, 1984b; Yamada and Ohyama, 1988; Smith et al, 1989; McConkey et al, 1988, 1989a and 1989b). The death is always in the thymus cortex, not in the medulla (Van Haelst, 1967a).

Intestinal mucosal cells in the lower third of the crypt (but not including the stem cells near the crypt base) occasionally undergo apoptosis spontaneously (Searle et al, 1975), and so are presumptively "primed". These are also the main targets for death by apoptosis in response to a diverse range of chemotherapeutic agents, ionising radiation and zinc deprivation (Ijiri and Potten, 1983 and 1987; Elmes, 1977).

Adrenal cortical epithelial cells of the innermost region of the gland, the zona reticularis, are exclusively the cells sensitive to apoptosis in response to ACTH

withdrawal (Wyllie et al, 1973a and 1973b), and low doses of the toxic carcinogen 7,12 DMBA (Currie et al, 1962). The zona reticularis includes the oldest post-mitotic cells in the adrenal cortex.

Spontaneous apoptosis is seen in late foetal and neonatal kidney cells in the rapidly proliferating nephrogenic zone in the outer renal cortex, but not in the zone of slow cell turnover in the medulla. Greatly increased cell deletion by apoptosis occurs in response to low to moderate doses of irradiation and follows the same spatial distribution (Gobe et al, 1988).

When antibodies become available for the proteins characteristic of primed cells, such as the endonuclease, it should be possible to visualise their distribution within tissues, and test these predictions more directly.

#### 4.5 MYC AND RAS CONTROL CELL FATE

Data from this thesis and from published studies on other experimental systems may be combined in a hypothetical model of genetic control of cell fate (figure 4.2). This scheme proposes that certain critical gene products regulate transit between states of **growth arrest, rapid turnover** of cells (both high proliferation and apoptosis), and **population expansion**. Gene products are positioned at sites of key action, however this does not exclude relevant effects at other sites. This scheme was developed together with Dive and Wyllie (1992) from a similar theme originally proposed to illustrate genetic control of B cell maturation in germinal follicles in lymph nodes with bcl-2 acting at the same site as ras. (Liu et al, 1990; Gregory et al, 1991).

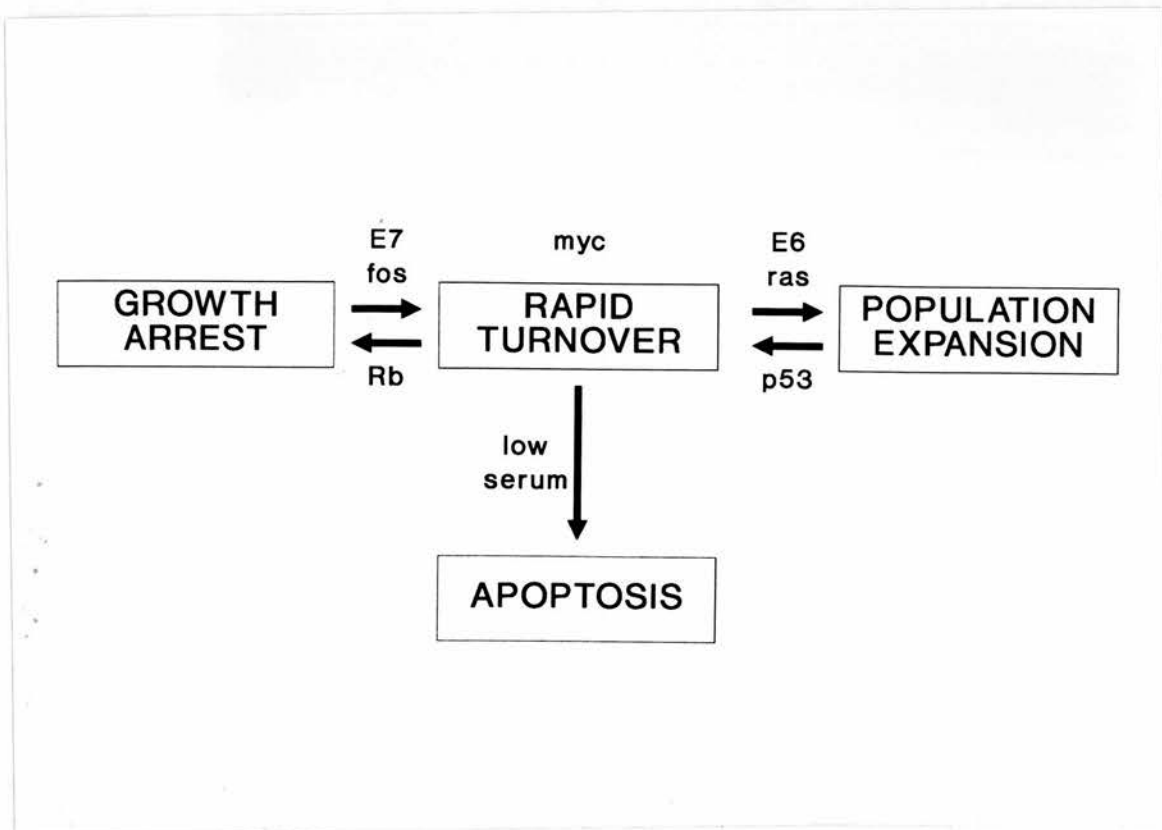


Figure 4.2

Hypothetical model that compounds data from a number of different cell systems, to illustrate potential regulation by certain genes of transit between stable states of growth arrest, rapid turnover of cells (high proliferation and high apoptosis), and population expansion. Some genes may act at other points as well as their key sites of action shown here. Other genes, not shown, may also act at these transition control points.



Cell transit between these states has been thoroughly researched. Growth arrest can be induced in cells by transfection with Rb (Huang et al, 1988). Growth arrest after treatment with TGF-beta is mediated by activation of Rb (Pietenpol et al, 1990; Moses et al, 1991). Exit from growth arrest to a state of proliferation is associated with activation of the immediate early response genes, which include the transiently expressed c-fos and egr-1, and persistently expressed c-myc (Sukhatme et al, 1988; Waters et al, 1990; Waters et al, 1991). Conversion of temperature sensitive p53 protein from mutated to wild type conformation induces proliferating fibroblasts to enter a high apoptotic state (Yonish-Rouach et al, 1991).

This thesis presents the association of c-myc with induction of priming for apoptosis within a population that is rapidly turning over. This is shown by expression of nuclear calcium/magnesium sensitive endonuclease activity that ladders chromatin, in a pattern characteristic of apoptosis. Triggering of apoptosis in these circumstances appears to be due to cell cycle block in the presence of myc activation. This is shown in rat fibroblasts containing a MYC-ER fusion protein vector that allows experimental manipulation of myc activity in cells arrested by a variety of means, including serum deprivation (Evan et al, 1992). Growth in very low serum of myc transfectants studied in this thesis (M7 and M8), also triggers high levels of apoptosis (E. Brown,

personal communication). It is possible that myc may have an independent role in triggering apoptosis, as there is a higher rate of apoptotic death in these myc transfectants, compared with the parent control, in the presence of high serum, but this requires further investigation. Experiments reported in this thesis strongly suggest that activated ras mediates transit of cells into the population expansion state by suppressing or reversing priming for apoptosis.

The diagram emphasises the pivotal role of c-myc in directing the rapid turnover state, which is the key control point of the pathway. C-myc drives the two coupled functions of proliferation and apoptosis. Downregulation of myc is required for growth arrest or differentiation (Evan et al, 1992). In contrast, population expansion requires the active suppression of apoptosis by a further signal, such as ras, which overrides myc. In the HPV transfectants studied in this thesis, E7 binding of Rb (possibly with consequent indirect myc activation) may stimulate transit to the rapid turnover state, and E6 mediated degradation of p53 may induce transit to the population expansion state, in a manner analagous to the function of ras in this pathway. In other cell systems bcl-2, the EBV gene LMP-1, SV40 large T antigen (LT), and adenovirus E1b may also act to directly suppress apoptosis or bind p53. Similarly, SV40 LT and adenovirus E1a bind Rb, and are predicted to stimulate transit to the rapid turnover

state.

The ability of ras to override myc, may explain the intermediate levels of apoptosis and population expansion of the combined myc plus ras cotransfectants MT9 and MT10. Precise rates of apoptosis may depend on the relative levels of the two proteins. Introduction of myc into an established ras transfectant (T1) to produce MT7 made little difference to its phenotype. The only exception was MT4, which was constructed by transfecting ras into the established myc transfectant M1. This would be predicted to suppress apoptosis. However, MT4 may express ras at a level too low to effectively reverse priming for apoptosis, as MT4 shows many phenotypic similarities to M1, its immediate parent line, including high apoptosis. These hypotheses could be tested by measurement of oncoprotein levels by ELISA.

Thus, myc appears to be the central regulator of cell fate, its downregulation influencing growth arrest and differentiation, and its expression inducing proliferation and apoptosis. However, the stimulus for apoptosis can be overridden by other products such as ras and HPV products.

## 5. **FINAL CONCLUSIONS**

To rationalise clinical and experimental data on tumour aggression, a small number of genes were selected for investigation of their relationships with neoplastic progression in the cervix and parameters of tumour growth.

1. There is a more rapid transition from CIN to cancer associated with HPV 18, indicated by higher CANCER/CIN prevalence ratios, compared with HPV 16. This may be partly explained by the lower levels of tumour cell apoptosis of HPV 18 transfectants, compared with those of HPV 16 transfectants. Lower apoptosis permits faster population expansion, thus increasing the risk of progression from precursor to malignancy.

2. Apoptosis appears to occur in two independent stages: priming and triggering. The induction of the effector proteins for apoptosis constitutes priming. The 3 myc transfectants express at least one of these, the nuclear calcium/magnesium sensitive endonuclease. In contrast, the parent cell, and one ras transfectant do not show this activity, whereas two other ras transfectants do so in a manner dependent on serum concentration. Induction of priming may explain the higher intrinsic rates of apoptosis of myc transfectants.

3. Constitutive expression of c-myc appears to regulate both proliferation and apoptosis, maintaining a stable state of rapid turnover. The suppression of apoptosis by activated ras produces transition to a state of population expansion. This contributes directly to formation of larger tumours by ras transfectants, and indirectly increases the risk of metastasis. HPV E6 and E7 proteins may influence transition between different states by binding cellular oncosuppressor proteins p53 and Rb.

## 6. BIBLIOGRAPHY

Aaronson SA. (1991). Growth factors and cancer. *Science* 254:1146-1153.

Abrams HD, Rohrschneider LR, Eisenman RN. (1982). Nuclear location of the putative transforming protein of avian myelocytomatosis virus. *Cell*. 29:427-439.

Agnantis NJ, Spandidos DA, Mahera H, et al. (1988). Immunohistochemical study of ras oncogene expression in endometrial and cervical human lesions. *Eur J Gynaec Oncol*. 9:360-365.

Albretsen C, Haukanes B-I, Aasland R, Kleppe K. (1988). Optimal conditions for hybridisation with oligonucleotides: a study with myc-oncogene DNA probes. *Anal Biochem*. 170:193-202.

Alitalo K, Ramsay G, Bishop JM, Pfeifer SO, Colby WW, Levinson AD. (1983a). Identification of nuclear proteins encoded by viral and cellular myc oncogenes. *Nature*. 306:274-277.

Alitalo K, Schwab, M, Lin CC, Varmus HE, Bishop JEM. (1983b). Homogeneously staining regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. *Proc Natl Acad Sci USA*. 80:1707-1711.

Alitalo K, Koskinen P, Makela TP, Saksela K, Sistonen L, Winqvist R. (1987). Myc oncogenes: activation and amplification. *Biochem Biophys Acta*. 907:1-32.

Alloub MI, Barr BBB, McLaren KM, Smith IW, Bunney MH, Smart GE. (1989). Human papillomavirus infection and cervical intraepithelial neoplasia in women with renal allografts. *Br Med J*. 298:153-156.

Alnemri ES, Litwack G. (1989). Glucocorticoid induced lymphocytolysis is not mediated by an induced endonuclease. *J Biol Chem*. 264:4104-4111.

Alonso T, Morgan RO, Marvizon JC, Zarbe H, Santos E. (1988). Malignant transformation by ras and other oncogenes produces common alterations in inositol phospholipid signalling pathways. *Proc Natl Acad Sci USA*. 85:4271-4275.

Andrejauskas E, Moroni C. (1989). Reversible abrogation of IL-3 dependence by an inducible H-ras oncogene. *EMBO J*. 8(9):2575-2581.

Androphy EJ, Hubbert NL, Schiller JT, Lowy DR. (1987). Identification of the HPV-16 E6 protein from transformed

- mouse cells and human cervical carcinoma cell lines. EMBO J. 6:989-992.
- Anonymous editorial. (1985). Genital Warts, human papillomaviruses and cervical cancer. Lancet. II:1045-1046.
- Anzano MA, Roberts AB, DeLarco JE, et al. (1985). Increased secretion of type B transforming growth factor accompanies viral transformation of cells. Mol Cell Biol. 5:242-247.
- Arends MJ, Morris RG, Wyllie AH. (1990a). Apoptosis: the role of the endonuclease. Am J Pathol. 136:593-608.
- Arends MJ, Wyllie AH, Bird CC. (1990b). Papillomaviruses and human cancer. Hum Pathol. 21:686-698.
- Arends MJ. (1991). Identification of HPV: in situ hybridization or polymerase chain reaction. J Pathol 164:191-193.
- Arends MJ, Wyllie AH. (1991). Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol. 32:223-254.
- Ashwell G, Hartford J. (1982). Carbohydrate specific receptors of the liver. Annu Rev Biochem. 51:531-554.
- Atkin NB, Baker MC. (1984). Non-random chromosome changes in carcinoma of the cervix uteri. II. Ten tumors in the triploid-tetraploid range. Cancer Genet Cytogenet. 13:189-207.
- Atkin NB. (1986). Chromosome changes in preneoplastic and neoplastic genital lesions. In: Origins of female genital cancer: virological and epidemiological aspects. Eds: Peto R, zur Hausen H. Banbury Report, Cold Spring Harbor Lab Press, N Y. 303-310.
- Baak JPA. (1990). Mitosis counting in tumors. Hum Pathol. 21:683-685.
- Baisch H, Beck H-P, Christensen IJ, et al. (1982). Comparison of the mathematical methods for the analysis of DNA histograms obtained by flow cytometry. Cell Tissue Kinet. 15:235-249.
- Baker CC. (1987). Sequence analysis of papillomavirus genomes. In: Salzman NP, Howley PM, eds. The Papovaviridae Volume 2: The Papillomaviruses. Plenum Press, New York/London. pp 321-385.
- Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. (1987). Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol. 61:962-971.



Baker SJ, Markowitz S, Fearon ER, Willson JKV, Vogelstein B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science*; 249:912-915.

Balkwill FR. (1989). Tumour necrosis factors. *Brit Med Bull.* 45:389-400.

Banks L, Spence P, Androphy E, et al. (1987). Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. *J Gen Virol.* 68:1351-1359.

Barbacid M. (1987). Ras genes. *Annu Rev Biochem.* 56:779-822.

Barbosa MS, Lowy DR, Schiller JT. (1989). Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J Virol.* 63:1404-1407.

Barbosa MS, Schlegel R. (1989). The E6 and E7 genes of HPV 18 are sufficient for inducing two stage in vitro transformation of human keratinocytes. *Oncogene.* 4:1529-1532.

Barbosa MS, Edmonds C, Fisher C, Schiller JT, Lowy DR, Vousden KH. (1990). The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO J.* 9:153-160.

Barker EA, Smuckler EA. (1973). Non-hepatic thioacetamide injury. I. Thymic cortical necrosis. *Am J Pathol.* 71:409-418.

Barnes W, Delgado G, Kurman RJ, et al. (1988). Possible prognostic significance of human papillomavirus type in cervical cancer. *Gynecol Oncol.* 29:267-273.

Barron BA, Richart RM. (1968). A statistical model of the natural history of cervical carcinoma based on a prospective study of 557 cases. *J Natl Cancer Inst.* 41:1343-1353.

Barry MA, Benke CA, Eastman A. (1990). Activation of programmed cell death (apoptosis) by cisplatin and other anticancer drugs, toxins and hypothermia. *Biochem Pharmacol.* 40:2352-2362.

Bar-Sagi D, Feramisco JR. (1985). Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. *Cell.* 42:841-848.

Batley J, Moulding C, Taub R, et al. (1983). The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt Lymphoma. *Cell.* 34:779-



Baxter GD, Collins RJ, Harmon BV, et al. (1989). Cell death by apoptosis in acute leukaemia. *J Pathol.* 158:123-129.

Beaudenon S, Kremsdorf D, Croissant O, Jablonska S, Wain-Hobson S, Orth G. (1986). A novel type of human papillomavirus associated with genital neoplasias. *Nature.* 321:246-249.

Beaudenon S, Kremsdorf D, Obalek S, et al. (1987). Plurality of genital human papillomaviruses: characterisation of two new types with distinct biological properties. *Virology.* 161:374-384.

Bedell MA, Jones KH, Laimins LA. (1987). The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH3T3 and Rat-1 cells. *J Virol.* 61:3635-3640.

Bedell MA, Jones KH, Grossman SR, Laimins LA. (1989). Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. *J Virol.* 63:1247-1255.

Bedell MA, Hudson JB, Golub TR, et al. (1991). Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. *J Virol.* 65:2254-2260.

Benda JA, Platz CE, Buchsbaum H, Lifshitz S. (1985). Mucin production in defining mixed carcinoma of the uterine cervix: a clinico-pathologic study. *Int J Gynecol Pathol.* 4:314-327.

Berkowitz RS, Ehrmann RL, Lavizzo-Mourey R, Knapp RC. (1979). Invasive cervical carcinoma in young women. *Gynecol Oncol.* 8:311-316.

Bernard H-U, Krammer G, Rowekamp WG. (1985). Construction of a fusion gene that confers resistance against Hygromycin B to mammalian cells in culture. *Exp Cell Res.* 158:237-243.

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM. (1983). Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumours. *EMBO J.* 2:2375-2383.

Bernstein SC, Weinberg RA. (1985). Expression of the metastatic phenotype in cells transfected with human metastatic tumour DNA. *Proc Natl Acad Sci USA.* 82:1726-1730.

Birchmeier W, Behrens J, Weidner KM, Frixen UH, Schipper J. (1991). Dominant and recessive genes involved in tumour invasion. *Curr Opin Cell Biol.* 3:832-840.

Bishop JM. (1983). Cellular oncogenes and retroviruses. *Annu Rev Biochem.* 52:301-354.

Bishop JM. (1991). Molecular themes in oncogenesis. *Cell.* 64:235-248.

Bonfiglio TA, Stoler MH. (1988). Human papillomavirus and cancer of the cervix. *Hum Pathol.* 19:621-622.

Bos JL, Fearon ER, Hamilton SR, et al. (1987). Prevalence of ras gene mutations in human colorectal cancers. *Nature.* 327:293-297.

Bourne HR, Sanders DA, McCormick F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature.* 348:125-132.

Bourne HR, Sanders DA, McCormick F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature.* 349:117-127.

Bowen ID, Bowen SM, Sarraf CE, Britton SL. (1988). Cell biology and malignant disease. In "The contribution of science to cancer medicine". Eds. Deeley TJ, Lloyd D, Sutherland WH. CRC Clifton House. 60-71.

Bowen ID, Bowen SM. (1990). Programmed cell death in tumours and tissues. Chapman and Hall, London.

Boyes DA, Fidler HK, Lock DR. (1963). The significance of in situ carcinoma of the uterine cervix. In: Proceedings of the First International Congress of Exfoliative Cytology. Philadelphia, J B Lippincott.

Brescia RJ, Jenson AB, Lancaster WD, Kurman RJ. (1986). The role of human papillomaviruses in the pathogenesis and histologic classification of precancerous lesions of the cervix. *Human Pathology.* 17:552-559.

Brodeur GM, Seeger RC, Schwab H, Varmus HE, Bishop JM. (1984). Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science.* 224:1121-1124.

Brown K, Quintanilla M, Ramsden M, Kerr IB, Yong S, Balmain A. (1986). V-ras genes from Harvey and BALB murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. *Cell.* 46:447-456.

Bubb V, McCance DJ, Schlegel R. (1988). DNA sequence of the HPV-16 E5 ORF and the structural conservation of its encoded protein. *Virology.* 163:243-246.

Buchkovich K, Duffy LA, Harlow E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell*; 58:1097-1105.

Buchmann A, Ruggeri B, Klein-Szanto AJP, Balmain A. (1991). Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-ras alleles on chromosome 7. *Cancer Res.* 57:4097-4101.

Buckley CH, Beards CS, Fox H. (1988). Pathological prognostic indicators in cervical cancer with particular reference to patients under the age of 40 years. *Br J Obstet Gynaecol.* 95:47-56.

Buckley CH, Fox H. (1989). Carcinoma of the cervix. *Recent Adv Histopath.* 14:63-78.

Bursch W, Lauer B, Timmermann-Trosiener I, Barthel G, Schuppler J, Schulte-Hermann R. (1984). Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumour promoters. *Carcinogenesis.* 5:453-458.

Buttayan R, Zakeri Z, Lockshin R, Wolgemuth D. (1988). Cascade induction of c-fos, c-myc, and heat shock 70k transcripts during regression of the rat ventral prostate gland. *Molecular Endocrinol.* 2:650-657.

Cameron GN. (1988). The EMBL database library. *Nucl Acids Res.* 16:1865-1867.

Campisi J, Gray HE, Pardee AB, Dean M, Sonenshein. (1983). Cell cycle control of c-myc but not c-ras expression is lost following chemical transformation. *Cell.* 26:241-247.

Cancer Registration Statistics Scotland 1978-1987. Cervix (ICD 180) Females. Information and Statistics Division, Common Services Agency, The Scottish Health Service. Edinburgh, 1990.

Capon DJ, Chen EY, Levinson AD, Seeburg PH, Goeddel DV. (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature.* 302:33-37.

Carey FA, Lamb D, Bird CC. (1990). Importance of sampling method in DNA analysis of lung cancer. *J Clin Pathol.* 43:820-823.

Cartwright RA, Sinson JD. (1980). Carcinoma of penis and cervix. *Lancet.* I:97.

Casas-Cordero M, Morin C, Roy M, Fortier M, Meisels A.

(1981). Origin of the koilocyte in condylomata of the human cervix: ultrastructural study. *Acta Cytol.* 25:383-392.

Chan W-K, Klock G, Bernard H-U. (1989). Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. *J Virol.* 63:3261-3269.

Chang AR. (1990). Carcinoma in situ of the cervix and its malignant potential. A lesson from New Zealand. *Cytopathology.* 1:321-328.

Chang EH, Furth ME, Scolnick EM, and Lowy DR. (1982). Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature.* 297:479-483.

Chen EY, Howley PM, Levinson D, Seeburg PH. (1982). The primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature.* 299:529-534.

Chesters PM, Vousden KH, Edmonds C, McCance DJ. (1990). Analysis of human papillomavirus type 16 open reading frame E7 immortalizing function in rat embryo fibroblast cells. *J Gen Virol.* 71:449-453.

Chin MT, Hirochika R, Hirochika H, Broker TR, Chow LT. (1988). Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. *J Virol.* 62:2994-3002.

Chittenden T, Livingston DM, Kaelin WG. (1991). The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* 65:1073-1082.

Choo K-B, Pan C-C, Han S-H. (1987). Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology.* 161:259-261.

Cleary ML, Smith SD, and Sklar J. (1986). Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell.* 47:19-28.

Coggin JR, zur Hausen H. (1979). Workshop on papillomaviruses and cancer. *Cancer Res.* 39:545-546.

Cohen JJ, Duke RC. (1984). Glucocorticoid activation of a calcium dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol.* 132:38-42.

Colbere-Garapin F, Horodniceanu F, Kourilsky P, Garapin A-C. (1981). A new dominant hybrid selective marker for higher eukaryotic cells. *J Mol Biol.* 150:1-14.

Colbert RA, Young DA. (1986). Glucocorticoid-induced messenger ribonucleic acids in rat thymic lymphocytes: Rapid primary effects specific for glucocorticoids. *Endocrinology.* 119:2598-2605.

Colby WW, Chen EY, Smith DH, Levinson AD. (1982). Identification of a nucleotide sequence of a human locus homologous to the v-myc oncogene of avian myelocytomatosis virus MC29. *Nature.* 301:722-725.

Cole MD. (1986). The myc oncogene: its role in transformation and differentiation. *Annu Rev Genet.* 20:361-384.

Cole MD. (1991). Myc meets its max. *Cell.* 65:715-716.

Cole ST, Danos O. (1987). Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome: phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. *J Mol Biol.* 193:599-608.

Collins S, Groudine, M. (1982). Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line. *Nature.* 298:679-681.

Collum RG, Alt FW. (1990). Are myc proteins transcription factors? *Cancer Cells.* 2:69-75.

Compton MM, Cidlowski JA. (1987). Identification of glucocorticoid-induced nuclease activity in thymocytes. A potential "lysis gene" product. *J Biol Chem.* 262:8288-8292.

Conroy LA, Jenkinson EJ, Owen JJT, Michell RH. (1991). The role of inositol lipid hydrolysis in the selection of immature thymocytes. *Biochem Soc Transact.* 19:905.

Cornelissen MTE, Smits ML, Briet MA, et al. (1990). Uniformity of the splicing pattern of the E6/E7 transcripts in human papillomavirus type 16 transformed human fibroblasts, human cervical premalignant lesions and carcinomas. *J Gen Virol.* 71:1243-1246.

Cotran RS, Kumar V, Robbins SL. (1989). Robbins pathologic basis of disease. 4th edition. WB Saunders, Philadelphia.

Cripe TP, Haugen TH, Turk JP, et al. (1987). Transcriptional regulation of the human papillomavirus 16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products:



implications for cervical carcinogenesis. EMBO J. 6:3745-3753.

Crook T, Storey A, Almond N, Osborn K, Crawford L. (1988). Human papillomavirus type 16 cooperates with activated ras and fos oncogenes in the hormone-dependent transformation of primary mouse cells. Proc Natl Acad Sci USA. 85:8820-8824.

Crook T, Greenfield I, Howard J, Stanley M. (1990). Alterations in growth properties of a human papillomavirus type 16 immortalized human cervical keratinocyte cell line correlates with amplification and overexpression of c-myc oncogene. Oncogene. 5:619-622.

Crook T, Wrede D, Tidy J, Scholefield J, Crawford L, Vousden KH. (1991). Status of c-myc, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. Onogene 6:1251-1257.

Crum CP, Mitao M, Levine RU, Silverstein S. (1985). Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. J Virol. 54:675-681.

Cullen AP, Reid R, Campion M, Lorincz AT. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J Virol. 65:606-612.

Currie AR, Hefelstein JE, Young S. (1962). Massive adrenal necrosis in rats caused by 9, 10-dimethyl-1, 2-benzanthracene and its inhibition by metyrapone. Lancet. II:1199-1200.

Debatin K-M, Goldmann CK, Bamford R, Waldmann TA, Krammer PH. (1990). Monoclonal antibody-mediated apoptosis in adult T-cell leukaemia. Lancet. II:497-500.

De Brux J, Orth G, Croissant O, Cochard B, Ionesco M. (1983). Condylomatous lesions of the cervix uteri: development in 2466 patients. Bull Cancer. 70:410-422.

DeCaprio JA, Ludlow JW, Figge J et al. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell. 54:275-283.

DeFeo-Jones P, Tatchell K, Robinson LC, Sigal IS, Vass WC, Lowy DR, Scolnick EM. (1985). Mammalian and yeast ras gene products: Biological function in their heterologous systems. Science. 228:179-184.

Deiss l, Kimchi A. (1991). A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory

signal. Science. 252:117-120.

Denekamp J, Hobson B. (1982). Endothelial cell proliferation in experimental tumours. Br J Cancer. 46:711-720.

Denzlinger C, Rapps S, Hagmann W, Keppler D. (1985). Leukotrienes as mediators in tissue trauma. Science. 230:330-332.

Der CJ, Krontiris TG, Cooper GM. (1982). Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci USA. 79:3637-3640.

Devereux J, Haeberli P, Smithies O. (1984). A comprehensive set of sequence-analysis programs for the VAX. Nucleic Acids Res. 12:387-395.

deVilliers E-M, Wagner D, Schneider A, et al. (1987). Human papillomavirus infections in women with and without abnormal cervical cytology. Lancet. II:703-705.

deVilliers E-M. (1989). Heterogeneity of the human papillomavirus group. J Virol. 63:4898-4903.

DiMaio D. (1991). Transforming activity of bovine and human papillomaviruses in cultured cells. Adv Cancer Res. 56:133-155.

DiPaolo JA, Woodworth CD, Popescu NC, Notario V, Doniger J. (1989). Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey ras. Oncogene. 4:395-399.

Dive C, Wyllie AH. (1992). Apoptosis and cancer chemotherapy. (manuscript submitted).

Donhuijsen K, Schmidt U, Hirche H, van Beuningen D, Budach V. (1990). Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol. 21:709-714.

Doorbar J, Campbell D, Grand RJA, Gallimore PH. (1986). Identification of the human papillomavirus 1a E4 gene products. EMBO J. 355-362.

Dotto GP, Parada LF, and Weinberg RA. (1985). Specific growth response of ras transformed embryo fibroblasts to tumour promoters. Nature. 318:472-475.

Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. (1990). Stimulation of p21ras upon T cell activation. Nature. 346:719-723.

- Drake M, Medley G, Mitchell H. (1987). Cytologic detection of human papillomavirus infection. *Obstet Gynecol Clin North Am.* 14:431-450.
- Dransfield I, Buckle A-M, Hogg N. (1990). Early events of the immune response mediated by leucocyte integrins. *Immunol Rev.* 114:29-44.
- Drickamer K. (1988). Two distinct classes of carbohydrate recognition domains in animal lectins. *J Biol Chem.* 263: 9557-9560.
- Duke RC, Chervenak R, Cohen JJ. (1983). Endogenous endonuclease - induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc Natl Acad Sci. USA.* 80:6361-6365.
- Duke RC, Cohen JJ. (1986). IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res.* 5:289-299.
- Durst M, Kleinheinz A, Hotz M, Gissmann L. (1985). The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. *J Gen Virol.* 66:1515-1522.
- Durst M, Croce CM, Gissmann L, Schwartz E, Huebner K. (1987a). Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proc Natl Acad Sci USA.* 84:1070-1074.
- Durst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig N E, Gissmann L. (1987b). Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene.* 1:251-256.
- Durst M, Gallahan D, Jay G, Rhim JS. (1989). Glucorticoid-enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and an activated ras oncogene. *Virology.* 173:767-771.
- Duvall E, Wyllie AH, Morris RG. (1985). Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology.* 56:351-358.
- Duvall E, Wyllie AH. (1986). Death and the cell. *Immunol Today.* 7:115-119.
- Dykes TA, McCall C, Weiner L, Duke R, Cohen JJ. (1987). Purification and characterisation of the endogenous endonuclease involved in programmed cell death of developing thymocytes. *Clinical Res.* 35:139A.
- Dyson JED, Simmons DM, Daniel J, McLaughlin JM, Quirke P, and Bird CC. (1986). Kinetic and physical studies of



- cell death induced by chemotherapeutic agents or hypothermia. *Cell Tissue Kinet.* 19:311-324.
- Dyson N, Howley PM, Munger K, Harlow E. (1990). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science.* 243:934-937.
- Edmonds C, Vousden KH. (1989). A point mutational analysis of human papillomavirus type 16 E7 protein. *J Virol.* 63:2650-2656.
- Eilers M, Picard D, Yamamoto KR, Bishop JM. (1989). Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature.* 340:66-68.
- Eisenman RN, Tachibana CY, Abrams HD, Hann SR. (1985). V-myc and c-myc encoded proteins are associated with the nuclear matrix. *Mol Cell Biol.* 5:114-126.
- Ellis PSJ, Whitehead R. (1981). Mitosis counting - a need for reappraisal. *Hum Pathol.* 12:3-4.
- Ellis RE, Yuan J, Horvitz R. (1991). Mechanisms and functions of cell death. *Ann Rev Cell Biol.* (in press).
- Elmes ME. (1977). Apoptosis in the small intestine of zinc-deficient and fasted rats. *J Pathol.* 123:219-223.
- Erikson J, Ar-Rushdi A, Drwinga HL, Nowell PC, Croce CM. (1983). Transcriptional activation of the translocated c-myc oncogene in Burkitt lymphoma. *Proc Natl Acad Sci USA.* 80:820-824.
- Evan GI, Hancock DC. (1985). Studies on the interaction of the human c-myc protein with the cell nucleus: p62<sup>myc</sup> as a member of a discrete subset of nuclear proteins. *Cell.* 43:253-261.
- Evan G, Wyllie A, Gilbert C, et al. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* (in press).
- Farnsworth A, Lavery C, Stoler MH. (1989). Human papillomavirus messenger RNA expression in adenocarcinoma in situ of the uterine cervix. *Int J Gynecol Pathol.* 8:321-330.
- Ferenczy A and Winkler B. (1987). Cervical intraepithelial neoplasia and condyloma. In: Blaustein's Pathology of the Female Genital Tract. Ed. Kurman R. 2nd ed. New York, Springer-Verlag pp 177-218.
- Ferguson DJP, Anderson TJ. (1981a). Ultrastructural observations on cell death by apoptosis in the resting

human breast. Virchows Arch (Pathol Anat). 393:193-203.

Ferguson DJP, Anderson TJ. (1981b). Morphological evaluation of cell turnover in relation to the menstrual cycle in the resting breast. Br J Cancer. 44:177-181.

Fesus L, Thomazy V, Falus A. (1987). Induction and activation of tissue transglutaminase during programmed cell death. FEBS Lett. 224:104-108.

Fesus L, Thomazy V, Autuori F, Ceru MP, Tarcsa E, Piacentini M. (1989). Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. FEBS Lett. 245:150-154.

Fesus L, Davies PJA, Piacentini M. (1991). Apoptosis: molecular mechanisms in programmed cell death. Eur J Cell Biol. 56:170-177.

Fidler IJ, Hart IR. (1982). Biological diversity in metastatic neoplasms: Origins and implications. Science. 217:998-1003.

Field JK, Spandidos DA. (1990). The role of ras and myc oncogenes in human solid tumours and their relevance in diagnosis and prognosis. Anti Cancer Res. 10:1-22.

Figge J, Smith TF. (1988). Cell-division sequence motif. Nature. 334:109.

Figge J, Webster T, Smith TF, Paucha E. (1988). Prediction of similar transforming regions in Simian Virus 40 large T, adenovirus E1A, and myc oncoproteins. J Virol. 62:1814-1818.

Filippovich IV, Sorokina NI, Soldatenkov VA, Romantzev EF, (1982). Supercoiled DNA repair in thymocyte fractions differing in radiosensitivity. Int J Radiat Biol. 42:31-44.

Finch JT, Klug A. (1976). Solenoidal model for superstructure in chromatin. Proc Natl Acad Sci (USA). 73:1897-1901.

Finlay CA, Hinds PW, Levine AJ. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell. 57:1083-1093.

Flieger D, Riethmuller G, Ziegler-Heitbrock HWL. (1989). Zn<sup>++</sup> inhibits both tumour necrosis factor-mediated DNA fragmentation and cytolysis. Int J Cancer. 44:315-319.

Fogh, J, Giovanella, JA. Eds. Nude mice in experimental and clinical research. Academic Press, New York. pp282-312.

- Folkman J. (1986). How is blood vessel growth regulated in normal and neoplastic tissue? *Cancer Res.* 46:467-473.
- Fukui Y, Kozasa T, Kaziro Y, Takeda T, Yamamoto M. (1986). Role for a ras homolog in the life cycle of *Schizosaccharomyces pombe*. *Cell.* 44:329-336.
- Galloway DA, McDougall JK. (1989). Human papillomaviruses and carcinomas. *Adv Virus Res.* 37:125-171.
- Gannon JV, Greaves R, Iggo R, Lane DP. (1990). Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.* 9:1595-1602.
- Giri I, Danos O. (1986). Papillomavirus genomes: from sequence data to biological properties. *Trends in Genetics.* 2:227-232.
- Gissmann L, Diehl V, Schultz-Coulon H-J, zur Hausen H. (1982). Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. *J Virol.* 44:393-400.
- Gissmann L. (1984). Papillomaviruses and their association with cancer in animals and in man. *Cancer Surveys.* 3:161-181.
- Gissmann L, Schneider A. (1986). Human papillomavirus DNA in preneoplastic and neoplastic genital lesions. In: *Origins of female genital cancer: virological and epidemiological aspects.* Eds: Peto R, zur Hausen H. Banbury Report, Cold Spring Harbor Lab Press, New York. pp217-224.
- Gius D, Grossman S, Bedell MA, Laimins LA. (1988). Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. *J Virol.* 62:665-672.
- Gloss B, Bernard HU, Seedorf K, Klock G. (1987). The upstream regulatory region of the human papillomavirus 16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. *EMBO J.* 6:3735-3743.
- Gloss B, Chong T, Bernard H-U. (1989). Numerous nuclear proteins bind the long control region of human papillomavirus type 16: a subset of 6 of 23 DNase I-protected segments coincides with the location of the cell-type-specific enhancer. *J Virol.* 63:1142-1152.
- Gobe GC, Axelsen RA, Harmon BV, Allan DJ. (1988). Cell death by apoptosis following x-irradiation of the foetal

and neonatal rat kidney. *Int J Radiat Biol.* 54:567-576.

Going JJ, Anderson TJ, Wyllie AH. (1992). Ras p21 in breast tissue: associations with pathology and cellular localisation. *Br J Cancer* 65:45-50.

Goldsborough M D, DiSilvestre D, Temple G F, Lorincz A T. (1989). Nucleotide sequence of human papillomavirus type 31: a cervical neoplasia-associated virus. *Virology.* 171:306-311.

Goldstein DJ, Finbow ME, Andresson T, McLean P, Smith K, Bubb V, Schlegel R. (1991). Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H<sup>+</sup>-ATPases. *Nature* 352:347-349.

Goyette M, Petropoulos CJ, Shank PK, and Fausto N. (1983). Expression of a cellular oncogene during liver regeneration. *Science.* 219:510-512.

Graham FL, Van Der Eb AJ. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52: 456-467.

Graham S, Priore R, Graham M, et al. (1979). Genital cancer in wives of penile cancer patients. *Cancer.* 44:1870-1874.

Green H. (1980). The keratinocyte as differentiated cell type. *Harvey Lectures, Series.* 74:101-155.

Gregory CD, Dive C, Henderson S, Smith CA, Williams GT, Gordon J, Rickinson AB. (1991). Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature.* 349:612-614.

Griffin NR, Dockey D, Lewis FA, Wells M. (1991). Demonstration of low frequency of HPV DNA in cervical adenocarcinoma and adenocarcinoma in situ by the PCR and in situ hybridisation. *Int J Gynecol Pathol.* 10:36-43.

Grossman SR, Laimins LA. (1989). E6 protein of human papillomavirus type 18 binds zinc. *Oncogene.* 4:1089-1093.

Grossman SR, Mora R, Laimins LA. (1989). Intracellular localisation and DNA binding properties of human papillomavirus type 18 E6 protein expressed with a Baculovirus vector. *J Virol.* 63:366-374.

Gullino P. (1980). The regression process in hormone-dependent mammary carcinomas. *Prog Cancer Res Ther.* 14:271-279.

Halbert CL, Demers GW, Galloway DA. (1991). The E7 gene of human papillomavirus type 16 is sufficient for

immortalization of human epithelial cells. J Virol. 65:473-478.

Hall PA, Levinson DA. (1990). Review: assessment of cell proliferation in histological material. J Clin Pathol. 43:184-192.

Hall PA, Levinson DA, Woods AL, et al. (1990). Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol. 162:285-294.

Halligan BD, Edwards KA, Liu LF (1985). Purification and characterisation of a type II DNA topoisomerase from bovine calf thymus. J Biol Chem. 260:2475-2482.

Hancock JF, Magee AI, Childs JE, Marshall CJ. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 57:1167-1177.

Handyside AH, Hunter S. (1986). Cell division and death in the mouse blastocyst before implantation. Roux's Arch Dev Biol. 195: 519-526.

Hann SR, Thompson CB, Eisenman RE. (1985). C-myc oncogene protein synthesis is independent of the cell cycle in human and avian cells. Nature. 314:366-368.

Hanselaar AGJM, Vooijs GP, Oud PS, Pahlplatz MMM, Beck JLM. (1988). DNA ploidy patterns in cervical intraepithelial neoplasia grade III with and without synchronous invasive squamous cell carcinoma. Cancer. 62:2537-2545.

Harrison DJ. (1988). Cell death in the diseased glomerulus. Histopathology. 12:679-683.

Haslett C, Savill JS, Meagher L. (1989). The neutrophil. Curr Opin Immunol. 2:10-18.

Henderson S, Rowe M, Gregory C, et al. (1991). Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell. 65:1107-1115.

Henson PM, Johnston RB. (1987). Tissue injury in inflammation. Oxidants, proteinases and cationic proteins. J Clin Invest. 79:669-674.

Herrlich P, Ponta H. (1989). "Nuclear" oncogenes convert extracellular stimuli into changes in the genetic program. Trends in Genetics. 5:112-116.

Hewish DR, Burgoyne LA. (1973). Chromatin substructure. The digestion of chromatin DNA at regularly spaced sites



by a nuclear deoxyribonuclease. Biochem Biophys Res Commun. 52:504-510.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 348:334-336.

Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. (1991). Bcl-2 protein is topographically restricted in tissues characterised by apoptotic cell death. Proc Natl Acad Sci USA. 88:6961-6965.

Howley PM, Yang Y-C, Spalholz BA, Rabson MS. (1986). Papillomavirus transforming functions. CIBA Foundation Symp. 120:39-52.

Huang AL, Ostrowski MC, Bernard D, Hager BL. (1981). Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumour virus. Cell. 27:245-255.

Huang H-JS, Yee J-K, Shew J-Y et al. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science. 242:1563-1566.

Hudson JB, Bedell MA, McCance DJ, Laimins LA. (1990). immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J Virol. 64:519-526.

Hynes RO. (1987). Integrins: a family of cell surface receptors. Cell. 48:549-554.

Ijiri K, Potten CS. (1983). Response of intestinal cells of differing topographical and heirarchical status to ten cytotoxic drugs and five sources of radiation. Br J Cancer. 47:175-185.

Ijiri K, Potten CS. (1987). Further studies on the response of intestinal crypt cells of different heirarchical status to eighteen different cytotoxic agents. Br J Cancer. 55:113-123.

Inoue T, Casanova HA, Morita K, Chihara T. (1986). The prognostic significance of the minimum thickness of uninvolved cervix in patients with cervical carcinoma stages IB, IIA and IIB. gynecol Oncol. 24:220-229.

Ireland D, Cole S, Kelly P, Monaghan JM. (1987). Mucin production in cervical intraepithelial neoplasia and in stage Ib carcinoma of cervix with pelvic lymph node metastases. Br J Obstet Gynaecol. 94:467-472.

Ishida R, Akiyoshi H, Takahoshi T. (1974). Isolation and

purification of calcium and magnesium-dependent endonuclease from rat liver nuclei. Biochem Biophys Res Commun. 56:703-710.

Itoh N, Yonehara S, Ishii A, et al. (1991). The polypeptide encoded by the cDNA for human cell surface antigen fas can mediate apoptosis. Cell. 66:233-243.

Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. (1990a). Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J Clin Pathol. 43:499-504.

Jackson DP, Payne J, Bell S, Lewis FA, Taylor GR, Peel KR, Sutton J, Quirke P. (1990b). Extraction of DNA from exfoliative cytology specimens and its suitability for analysis by the polymerase chain reaction. Cytopathology. 1:87-96.

Jakobsen A, Kristensen P B, Poulsen H K. (1983). Flow cytometric classification of biopsy specimens from cervical intraepithelial neoplasia. Cytometry. 4:166-169.

Johnson PW, Baubock C, Roder JC. (1985). Transfection of a rat cell line with the v-Ki-ras oncogene is associated with enhanced susceptibility to natural killer cell lysis. J Exp Med. 162:1732-1735.

Jones B, Camplejohn RS. (1983). Stathmokinetic measurement of tumour cell proliferation in relation to vascular proximity. Cell Tiss Kinet. 16:351-355.

Jurnak F, Heffron S, Bergmann E. (1990). Conformational changes in the activation of ras p21: implications for related proteins. Cell. 60:525-528.

Kagiyama A, Savage HE, Michael LH, et al. (1989). Molecular basis of complement activation in ischemic myocardium: identification of specific molecules of mitochondrial origin that bind human C1q and fix complement. Circ Res. 64:607-615.

Kahn T, Schwartz E, zur Hausen H. (1986). Molecular cloning and characterization of the DNA of a new human papillomavirus (HPV30) from a laryngeal carcinoma. Int J Cancer. 37:61-65.

Kakkis E, Riggs KJ, Gillespie W, Calame K. (1989). A transcriptional repressor of c-myc. Nature. 339:718-721.

Kanda T, Watanabe S, Yoshiike K. (1988). immortalization of primary rat cells by human papillomavirus type 16 subgenomic fragments controlled by the SV40 promoter. Virology. 165:321-325.

Kasid A, Lippman MC, Papageorge AG, Lowy DR, Gelman EP.

(1985). Transfection of v-Ha-ras1 DNA into MCF-7 breast cancer cells bypasses dependence on oestrogen for tumorigenicity. *Science*. 228:725-728.

Kaster KR, Burgett SS, Rao RN, Ingolia TD. (1983). Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. *Nucleic Acids Res*. 11:6895-6911.

Kato M, Toguchida J, Hondo K, et al. (1990). Elevated frequency of a specific allele of the c-myc gene in male patients with bone and soft tissue sarcomas. *Int J Cancer*. 45:47-49.

Katoaka T, Povors S, McGill C, et al. (1984). Genetic analysis of yeast RAS 1 and RAS 2 genes. *Cell*. 37:437-445.

Kaur P and McDougall JK. (1989). HPV 18 immortalization of human keratinocytes. *Virology*. 173:302-310.

Kaur P, McDougall JK, Cone R. (1989). Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. *J Gen Virol*. 70:1261-1266.

Keath AJ, Caimi PG, Cole MD. (1984). Fibroblast lines expressing activated c-myc oncogenes are tumorigenic in nude mice and syngeneic animals. *Cell*. 39:339-348.

Kelly K, Cochran BH, Stiles CD, Leder P. (1983). Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. *Cell*. 35:603-610.

Kerr JFR. (1971). Shrinkage necrosis: a distinct mode of cellular death. *J Pathol*. 105:13-20.

Kerr JFR, Searle J. (1972a). A suggested explanation for the paradoxically slow growth rate of basal cell carcinomas contain numerous mitotic figures. *J Pathol*. 107:41-44.

Kerr JFR, Searle J. (1972b). The digestion of cellular fragments within phagolysosomes in carcinoma cells. *J Pathol*. 108:55-59

Kerr JFR, Wyllie AH, Currie AR. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26:239-257.

Kerr JFR, Searle J. (1973). Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch (B) (Cell Pathol)*. 13:87-102.

Kerr JFR, Harmon B, Searle J. (1974). An electron-



microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibres. *J Cell Sci.* 14:571-585.

Kerr KM, Lamb D. (1984). Actual growth rate and tumour cell proliferation in human pulmonary neoplasms. *Br J Cancer.* 50:343-349.

Kessler II. (1977). Venereal factors in human cervical cancer: Evidence from marital clusters. *Cancer.* 39:1912-1919.

Kitayama H, Sugimoto Y, Matzuzaki T, Ikawa Y, Noda M. (1989). A ras-related gene with transformation suppressor activity. *Cell.* 56:77-84.

Kiyabu M T, Shibata D, Arnheim N, Martin J W, Fitzgibbons P L. (1989). Detection of human papillomavirus in formalin-fixed, invasive squamous carcinomas using the polymerase chain reaction. *Am J Surg Pathol.* 13:221-224.

Klausner JM, Paterson IS, Goldman G, Kobzik L, Rodzen C, Lawrence R, Valeri CR, Shepro D, Hechtman HB. (1989). Postischemic renal injury is mediated by neutrophils and leukotrienes. *Am J Physiol.* 256 (Renal Fluid Electrolyte Physiol. 25): F794-F802.

Klein G. (1983). Specific chromosome translocations and the genesis of B-cell derived tumours in mice and men. *Cell.* 32:311-315.

Klostergaard J, Foster WA, Hamilton DA, Turpin J, Lopez-Berenstein G. (1986). Effector mechanisms in human monocyte-mediated tumour cytotoxicity in vivo: Biochemical, functional and serological characterization of cytotoxins produced by peripheral blood monocytes isolated by counterflow elutriation. *Cancer Res.* 46:2871-2875.

Knudson AG. (1971). Mutation and cancer: Statistical study of retinoblastoma. *Proc Natl Acad Sci USA.* 68:820-823.

Kornfield R, Kornfield S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem.* 54:631-664.

Koskinen PJ, Sistonen L, Evan G, Morimoto R, Alitalo K. (1991). Nuclear co-localization of cellular and viral myc proteins with HSP70 in myc overexpressing cells. *J Virol.* 65:842-851.

Koss LG, Durfee GR. (1956). Unusual patterns of squamous epithelium of uterine cervix: cytologic and pathologic study of koilocytotic atypia. *Ann N Y Acad Sci.* 63:1245-

1261.

Koss LG, Phillips A. (1974). Summary and recommendations of the workshop on uterine cervical cancer. *Cancer*. 33:1753-1754.

Kottmeier H. (1961). Evolution et traitement des epitheliomas. *Rev Franc Gynecol*. 56:821-826.

Koufos A, Hansen M F, Lampkin B C, et al. (1984). Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumour. *Nature*. 309:170-172.

Kreider J W, Howett M K, Wolfe S A, et al. (1985). Morphological transformation in vivo of human uterine cervix with papillomavirus from condylomata acuminata. *Nature*. 317:639-641.

Kurman R J, Schiffman M H, Lancaster W D, et al. (1988). Analysis of individual papillomavirus types in cervical neoplasia: a possible role for type 18 in rapid progression. *Am J Obstet Gynecol*. 159:293-296.

Kwok S, Higuchi R. (1989). Avoiding false positives with PCR. *Nature*. 339:237-238.

Kyprianou N, English HF, Isaacs JT. (1990). Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. *Cancer Res*. 50:3748-3753.

Laiho M, DeCaprio JA, Ludlow JW, Livingstone DM, Massague J. (1990). Growth inhibition by TGF- $\beta$  linked to suppression of retinoblastoma protein phosphorylation. *Cell* 62:175-185.

Lala PK. (1972). Evaluation of the mode of cell death in Ehrlich ascites tumor. *Cancer*. 29:261-266.

Lambert P F, Spalholz B A, Howley P M. (1987). A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. *Cell*. 50:69-78.

Land H, Parada LF, Weinberg RA. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*. 304:596-602.

Lane DP, Crawford LV. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature*. 278:261-263.

Lee W-H, Shew J-Y, Hong FD et al. (1987). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* 329:642-645.

Lee WMF, Schwab M, Varmus HE. (1985). Augmented expression of normal c-myc is sufficient for cotransformation of rat embryo cells with a mutant ras gene. Mol Cell Biol. 5:3345-3356.

Lehn H, Krieg P, Sauer G. (1985). Papillomavirus genomes in human cervical tumors: analysis of their transcriptional activity. Proc Natl Acad Sci USA. 82:5540-5544.

Lehn H, Villa L L, Marziona F, Hilgarth M, Hillemans H-G, Sauer G. (1988). Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract. J Gen Virol. 69:187-196.

Lenoir GM, Bornkam GW. (1987). Burkitt's lymphoma, a human cancer model for the study of the multistep development of cancer: proposal for a new scenario. In "Advances in Viral Oncology". Ed. Klein G. Raven Press, New York pp 173-205.

Levi JE, Delcelo R, Alberti VN, et al. (1989). Human papillomavirus DNA in respiratory papillomatosis detected by in situ hybridisation and polymerase chain reaction. Am J Pathol. 135:1179-1184.

Lewin B. (1990). Driving the cell cycle: M Phase kinase, its partners and substrates. Cell. 61:743-752.

Liotta LA. (1988). Gene products which play a role in cancer invasion and metastasis. Breast Cancer Research and Treatment. 11:113-124.

Lipp M, Schilling R, Bernhardt G. (1989). Transactivation of human MYC: the second promotor is target for the stimulation by adenovirus Ela proteins. Oncogene. 4:535-541.

Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD. (1983). Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature. 306:194-196.

Liu LF, Liu CC, Alberts BM. (1980). Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break. Cell. 19:697-707.

Liu Y-J, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan ICM. (1989). The mechanism of antigen-driven selection in germinal centres. Nature. 342:929-931.

Lo Y-MD, Mehal WZ, Fleming KA. (1989). In vitro amplification of hepatitis B virus sequences from liver tumour DNA and from paraffin wax embedded tissues using

- the polymerase chain reaction. *J Clin Pathol.* 42:840-846.
- Loke S-L, Neckars LM, Schwab G, Jaffe ES. (1988). C-myc protein in normal tissue. Effects of fixation on its apparent subcellular distribution. *Am J Pathol.* 131:29-37.
- Lorincz A T, Lancaster W D, Temple G F. (1986). Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. *J Virol.* 58:225-229.
- Lorincz A T, Quinn A P, Lancaster W D, Temple G F. (1987a). A new type of papillomavirus associated with cancer of the uterine cervix. *Virology.* 159:187-190.
- Lorincz A T, Temple G F, Kurman R J, Jenson A B, Lancaster W D. (1987b). Oncogenic association of specific human papillomavirus types with cervical neoplasia. *J Natl Cancer Inst.* 79:671-676.
- Lotem J, Cragoe EJ, Sachs L. (1991). Rescue from programmed cell death in leukaemic and normal myeloid cells. *Blood* 78:953-960.
- Lowy DR, Willumsen BM. (1989). New clue to ras lipid glue. *Nature.* 341:384-385.
- Lusky M, Botchan M R. (1985). Genetic analysis of bovine papillomavirus type 1 trans-acting replication factors. *J Virol.* 53:955-965.
- Ludlow JW, De Caprio JA, Huang C-M, Lee W-H, Paucha E, Livingstone DM. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell.* 56:57-65.
- Maniatis T, Fritsch EF, Sambrook J. (1982). *Molecular Cloning. A laboratory manual.* Cold Spring Harbor Laboratory. 1st Edition.
- Manos M, Lee K, Greer C, Waldman J, Kiviat N, Holmes K, Wheeler C. (1990). Looking for human papillomavirus type 16 by PCR. *Lancet.* I:734.
- Marshall CJ, Vousden K, Ozanne B. (1985). The involvement of activated ras in determining the transformation phenotype. *Proc Roy Sci (B).* 226:99-106.
- Martin P, Vass WC, Schiller JT, Lowy DR, Velu TJ. (1989). The bovine papillomavirus E5 transforming protein can stimulate the transforming ability of EGF and CSF-1 receptors. *Cell.* 59:21-32.
- Martz E, Steinberg MS. (1973). Contact inhibition of

what? An analytical review. J Cell Physiol. 81:25-37.

Martz E, Howell DM. (1989). CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. Immunol Today. 10:79-86.

Matlaszewski G, Schneider J, Banks L, Jones N, Murray A, Crawford L. (1987). Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J. 6:1741-1746.

Matlaszewski G, Osborn K, Banks L, Stanley M, Crawford L. (1988). Transformation of primary human fibroblast cells with human papillomavirus type 16 DNA and EJ-ras. Int J Cancer. 42:232-238.

Matsukura T, Kanda T, Furuno A, Yoshikawa H, Kawana T, Yoshiike K. (1986). Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J Virol. 58:979-982.

Matsukura T, Koi S, Sugase M. (1989). Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. Virology. 172:63-72.

McCance D J, Kopan R, Fuchs E, Laimins L A. (1988). Human papillomavirus type 16 alters human epithelial cell differentiation in vitro. Proc Natl Acad Sci USA. 85:7169-7173.

McConkey DJ, Hartzell P, Duddy SK, Hakansson H, Orrenius S. (1988). 2, 3, 7, 8 - Tetrachlorodibenzo-p-toxin kills immature thymocytes by  $Ca^{2+}$ -mediated endonuclease activation. Science. 242:256-259.

McConkey DJ, Nicotera P, Hartzell P, Bolloma G, Wyllie AH, Orrenius S. (1989a). Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic  $Ca^{2+}$  concentration. Arch Biochem Biophys. 269:365-370.

McConkey DJ, Hartzell P, Amador-Perez JF, Orrenius S, Jondal M.J. (1989b). Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. J Immunol. 143:1801-1806.

McConkey DJ, Hartzell P, Jondal M, Orrenius S. (1989c). Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. J Biol Chem. 264:13399-13402.

McCormick F. (1989). Ras GTPase activating protein: signal transmitter and signal terminator. Cell. 56:5-8.



McDonnell TJ, Deanne N, Platt FM, Nunez G, Jaeger U, McKearn JP, Korsmeyer SJ. (1989). Bcl-2 immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell*. 57:79-82.

McDonnell TJ, Korsmeyer SJ. (1991). Progression from lymphoid hyperplasia to high grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* 349:254-256.

McDougall JK. (1990). HPV immortalization and transformation. *PV Report*. 1(3):1-4.

Meisels A, Morin C. (1986). Flat condyloma of the cervix: two variants with different prognosis. In: *Viral etiology of cervical cancer*. Eds. Peto R, zur Hausen H. (Banbury Report No. 21). Cold Spring Harbor NY: CSH Press pp 115-119.

Meisels A, Morin C, Casas-Cordero M. (1982). Human papillomavirus infection of the uterine cervix. *Int J Gynec Pathol*. 1:75-94.

Melchers W, van den Brule A, Walboomers J, et al. (1989). Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and southern blot analysis. *J Med Virol*. 27:329-335.

Meyers C, Laimins LA. (1992). In vitro model systems for the study of HPV-induced neoplasias. *Papillomavirus Report*. 3(1):1-3.

Milner J, Watson JV. (1990). Addition of fresh medium induces cell cycle and conformation changes in p53, a tumour suppressor protein. *Oncogene*. 5:1683-1690.

Moberg PJ, Einhorn N, Silfversward C, Soderberg G. (1986). Adenocarcinoma of the uterine cervix. *Cancer*. 57:407-410.

Montpetit ML, Lawless KR, Tenniswood M. (1986). Androgen-repressed messages in the rat ventral prostate. *Prostate*. 8:25-36.

Moore JV. (1983). Cytotoxic injury to cell populations of solid tumours. In "*Cytotoxic Insult to Tissue*". Eds. Potten CS, Hendry JH. Churchill Livingstone, Edinburgh. 368-404.

Moore JV. (1987). Death of cells and necrosis of tumours. In: "*Perspectives in mammalian cell death*". Ed. Potten CS. Oxford University Press, Oxford. pp295-325.

Morasca L, Erba E. (1986). Flow Cytometry. In: *Animal Culture: A Practical Approach*. Ed. Freshney RI. IRL Press.

Morin C, Braun L, Casas-Cordero M, et al. (1981). Confirmation of the papillomavirus etiology of condylomatous cervix lesions by the peroxidase-antiperoxidase technique. J Natl Cancer Inst. 66:831-835.

Morris BJ, Flanagan JL, McKinnon KJ, Nightingale BN. (1988). Papillomavirus screening of cervical lavages by polymerase chain reaction. Lancet. II:1368.

Morris RG, Arends MJ, Bishop P, Sizer K, Duvall E, Bird CC. (1990). Sensitivity of digoxigenin and biotin labelled probes for detection of HPV by in situ hybridisation. J Clin Pathol. 43:800-805.

Morris RG, Duvall E, Hargreaves AD, and Wyllie AH. (1984). Hormone-induced cell death. 2. Surface changes in thymocytes undergoing apoptosis. Am J Pathol. 115:426-436.

Morris RG, Hargreaves AD, Duvall ED, Wyllie AH. (1984). Hormone-induced cell death. 2. Surface changes in thymocytes undergoing apoptosis. Am J Pathol. 115:426-436.

Moses HL, Yuang EY, Pietsenpol JA. (1990). TGFbeta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell. 63:245-247.

Mougneau E, Lemieux B, Rassoulzadegan M, Cuzin F. (1984). Biological activities of v-myc and rearranged c-myc oncogenes in rat fibroblast cells in culture. Proc Natl Acad Sci USA. 81:5758-5762.

Mulcahy LS, Smith MR, Stacey DW. (1985). Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature. 313:241-243.

Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. (1989a). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol. 63:4417-4421.

Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. (1989b). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 8:4099-4105.

Munger K, Yee CL, Phelps WC, et al. (1991). Biochemical and biological differences between E7 oncoproteins of the high and low risk human papillomavirus types are determined by amino terminal sequences. J Virol. 65:3943-3948.

Munoz N, Bosch X, Kaldor JM. (1987). Does human

- papillomavirus cause cervical cancer? The state of the epidemiological evidence. *Br J Cancer*. 57:1-5.
- Munoz N, Bosch FX. (1991). Current views on the role of human papillomavirus in the genesis of cervical neoplasia. *Papillomavirus Report*. 2(3):57-60.
- Muschel RJ, Williams JE, Lowy DR, Liotta LA. (1985). Harvey ras induction of metastatic potential depends upon oncogene activation and the type of recipient cell. *Am J Pathol*. 121:1-8.
- Naghashfar ZS, Rosenshein NB, Lorincz AT, Buscema J, Shah KV. (1987). Characterization of human papillomavirus type 45, a new type 18-related virus of the genital tract. *J Gen Virol*. 68:3073-3079.
- Nakamura M, Sakaki Y, Watanabe N, Takagi Y. (1981). Purification and characterisation of the  $Ca^{2+}$  plus  $Mg^{2+}$  dependent endodeoxyribonuclease from calf thymus chromatin. *J Biochem*. 89:143-152.
- National Cancer Institute Workshop. (1988). The 1988 Bethesda System for reporting cervical/vaginal cytologic diagnoses. *Hum Pathol*. 21:704-708.
- Ng S-Y, Gunning P, Eddy R, et al. (1985). Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of non-coding regions and chromosomal dispersion of pseudogenes. *Mol Cell Biol*. 5:2720-2732.
- Noda M, Ko M, Ogura A, et al. (1985). Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature*. 318:73-75.
- Norris HJ. (1976). Mitosis counting III. *Hum Pathol*. 7:483-484.
- Nowell PC. (1976). The clonal evolution of tumour cell populations. *Science*. 194:23-28.
- Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D. (1983). The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucl Acids Res*. 11:1759-1771.
- Nunez G, London L, Hockenberry D, Alexander M, McKearn JP, Korsmeyer SJ. (1990). Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived haemopoietic cell lines. *J Immunol*. 144:3602-3610.
- Ocadiz R, Saucedo R, Cruz M, Graef A, Gariglio P. (1987). High correlation between molecular alterations of the c-myc oncogene and carcinoma of the uterine cervix. *Cancer Res*. 47:4173-4177.



- O'Connor TM, Wytttenbach CR. (1974). Cell death in the embryonic chick spinal cord. *J Cell Biol.* 60:448-459.
- Ohyama H, Yamada T, Watanabe I. (1981). Cell volume reduction associated with interphase death in rat thymocytes. *Radiat Res.* 85:333-339.
- Okagaki T, Tase T, Twiggs LB, Carson LF. (1989). Histogenesis of cervical adenocarcinoma with reference to human papillomavirus 18 as a carcinogen. *J Reproduct Med.* 34:639-644.
- Old LJ. (1985). Tumour necrosis factor. *Science.* 230:630-632.
- Olof P, Hellberg A, Kallskog TOK. (1989). Neutrophil-mediated post-ischemic tubular leakage in the rat kidney. *Kidney International.* 36:555-561.
- O'Shea JD, Hay MF, Cran DG. (1978). Ultrastructural changes in the theca interna during follicular atresia in sheep. *J Reprod Fertil.* 54:183-187.
- Owens GP, Hahn WE, Cohen JJ. (1991). Identification of mRNAs associated with programmed cell death in immature thymocytes. *Mol Cell Biol.* 11:4177-4188.
- Ozanne B, Wheeler T, Kaplan PL. (1982). Cells transformed by RNA and DNA tumour viruses produce transforming factors. *Fed Proc.* 41:3004-3007.
- Parada LF, Tabin CJ, Shih S, Weinberg RA. (1982). Human EJ bladder carcinoma oncogene is homologous of Harvey sarcoma virus ras gene. *Nature.* 297:474-478.
- Pater MM, Pater A. (1985). Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. *Virology.* 145:313-318.
- Pater MM, Dunne J, Hogan G, Ghatage P, Pater A. (1986). Human papillomavirus types 16 and 18 sequences in early cervical neoplasia. *Virology.* 155:13-18.
- Pater MM, Hughes GA, Hyslop DE, Nakshatri H, Pater A. (1988a). Glucocorticoid-dependent oncogenic transformation by type 16 but not type 11 human papillomavirus DNA. *Nature.* 335:832-835.
- Pater MM, Pater A. (1988b). Expression of human papillomavirus types 16 and 18 DNA sequences in cervical carcinoma cell lines. *J Med Virol.* 26:185-195.
- Penn LJZ, Brooks MW, Laufer EM, Land H. (1990). Negative autoregulation of c-myc transcription. *EMBO J.* 9:1113-1121.

Pettersson U, Ahola H, Stenlund A, Moreno-Lopez J. (1987). Organization and expression of papillomavirus genomes. In: Salzman N P, Howley P M. eds. The papovaviridae Volume 2: The papillomaviruses. Plenum Press, New York/London. 67-107.

Petti L, Nilson L, DiMaio D. (1991). Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J 10:845-855.

Pfister H. (1984). Biology and biochemistry of papillomaviruses. Rev Physiol Biochem Pharmacol. 99:111-181.

Pfister H, Krubke J, Dietrich W, Iftner T, Fuchs PG. (1986). Classification of the papillomaviruses - mapping the genome. In: Papillomaviruses - Ciba Foundation Symposium 120. Eds, D Evered, S Clark. John Wiley and sons, Chichester, New York, Brisbane, Toronto, Singapore. 3-14.

Pfister H. (1987a). Current papillomavirus classification scheme. Sixth International Papillomavirus Workshop: short review of current literature. 1-10.

Pfister H. (1987b). Human papillomaviruses and genital cancer. Adv Cancer Res. 48:113-147.

Phelps WC, Howley PM. (1987). Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. J Virol. 61:1630-1638.

Phelps WC, Yee CL, Munger K, Howley PM. (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell. 53:539-547.

Phillips DR, Charo IF, Parise LV, Fitzgerald LA. (1988). The platelet membrane glycoproteins IIb-IIIa complex. Blood. 71: 831-843.

Pichon F, Seuwen K, Pouyssegur J, Lagarde AE. (1988). Progressive relaxation of Go-arrest controls and altered responsiveness to insulin, EGF and thrombin in CCL39 lung fibroblasts overexpressing myc and ras oncogenes. Oncogene. 3:373-381.

Pietenpol JA, Stein RW, Moran E et al. (1990). TGF- $\beta$ 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell. 61:777-785.

Pilarski WP, Glassman DL, Krzyzek RA, Sadowski PL, Robbins AK. (1984). Cloning and expression in Escherichia

- coli of the bovine papillomavirus L1 and L2 open reading frames. *Biotechnology*. 1:356-360.
- Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA. (1987). Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J Virol*. 61:1061-1066.
- Pirisi L, Creek KE, Doniger J, DiPaolo JA. (1988). Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus DNA. *Carcinogenesis*. 9:1573-1579.
- Pizon V, Chardin P, Lerosay I, Olofsson B, Tavitian A. (1988). Human cDNAs rap1 and rap2 homologous to the Drosophila gene Dras3 encode proteins closely related to ras in the effector region. *Oncogene*. 3:201-204.
- Popescu NC, DiPaola JA. (1989). Preferential sites for viral integration on mammalian genome. *Cancer Genet Cytogenet*. 42:157-171.
- Popescu NC, DiPaolo JA, Amsbaugh SC. (1987). Integration sites of human papillomavirus type 18 DNA sequences on HeLa cell chromosomes. *Cytogenet Cell Genet*. 44:58-62.
- Popescu NC, DiPaolo JA. (1990). Integration of human papillomavirus 16 DNA and genomic rearrangements in immortalized human keratinocyte lines. *Cancer Res*. 50:1316-1323.
- Pozzatti R, Muschel R, Williams J. (1986). Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. *Science*. 232:223-227.
- Pragnell IB, Spandidos DA, Wilkie NM. (1985). Consequences of altered oncogene expression in rodent cells. *Proc Roy Soc. (B)*. 226:107-119.
- Prempre T, Amornmarn R, Wizenberg MJ. (1985). A therapeutic approach to primary adenocarcinoma of the cervix. *Cancer*. 56:1264-1268.
- Quade K. (1979). Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. *Virology*. 98:461-465.
- Quinn CM, Wright NA. (1990). The clinical assessment of proliferation and growth in human tumours: evaluation of methods and applications as prognostic variables. *J Pathol*. 160:93-102.
- Quintanilla M, Brown K, Ramsden M, Balmain A. (1986). Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature*. 322:78-80.

Rader JS, Golub TR, Hudson JB, Patel D, Bedell MA, Laimins LA. (1990). In vitro differentiation of epithelial cells from cervical neoplasias resembles in vivo lesions. *Oncogene*. 5:571-576.

Reddy EP, Reynolds RK, Santos E, Barbacid M. (1982). A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature*. 300:149-152.

Resnick RM, Cornelissen MT, Wright DK, et al. (1990). Detection and typing of HPV in archival cervical cancer specimens by DNA amplification with consensus primers. *J Natl Cancer Inst*. 82(18):1477-1484.

Report of the Task Force of the Department of Health and Welfare of Canada. Cervical Cancer Screening Programs. The Walton Report (1982). *Can Med Assoc J*. 127:581-589.

Richart RM. (1973). Cervical intraepithelial neoplasia. In: *Pathology Annual*. Ed. Sommers SC. New York, Appleton-Century-Crofts, pp 301-328.

Richart RM and Barron BA. (1969). A follow-up study of patients with cervical dysplasia. *Am J Obstet Gynecol*. 105:386-393.

Richart RM. (1987). Causes and management of cervical intraepithelial neoplasia. *Cancer*. 60:1951-1959.

Rickinson AB. (1990). Immunological aspects of virus-associated human tumours. *Cancer Cells*. 2:287-291.

Riou G, Barrois M, Tordjman I, Dutronquay V, Orth G. (1984). Presence de genomes de papillomavirus et amplification des oncogenes c-myc et c-Ha-ras dans des cancers envahissants du col de l'uterus. *C R Acad Sc Paris*. 299 sIII(14):575-580.

Riou G, Barrois M, Le M, George M, Le Doussal V, Haie C. (1987). C-myc proto-oncogene expression and prognosis in early carcinoma of the uterine cervix. *Lancet*. I:761-763.

Riou G, Barrois M, Sheng Z-M, Duvillard P, Lhomme C. (1988). Somatic deletions and mutations of c-Ha-ras gene in human cervical cancers. *Oncogene*. 3:329-333.

Riou G, Favre M, Jeannel D, Bourhis J, LePoussal V, Orth G. (1990). Association between poor prognosis in early stage invasive carcinomas and non-detection of HPV DNA. *Lancet*. II:1171-1174.

Robbins PD, Horowitz JM, Mulligan RC. (1990). Negative regulation of human c-fos expression by the

retinoblastoma gene product. Nature. 346-668-671.

Robertson M. (1985). Message of myc in context. Nature. 309:585-587.

Romanczuk H, Villa LL, Schlegel R, Howley PM. (1991). The viral transcriptional regulatory region upstream of the E6 and E7 genes is a major determinant of the differential immortalization activities of human papillomavirus types 16 and 18. J Virol. 65:2739-2744.

Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation. 67:1016-1023.

Rotello RJ, Hocker MB, Gerschenson LE. (1989). Biochemical evidence for programmed cell death in rabbit uterine epithelium. Am J Pathol. 134:491-495.

Rotin D, Robinson B, Tannock IF. (1986). Influence of hypoxia and an acid environment on the metabolism and viability of cultured cells: Potential implications for cell death in tumours. Cancer Res. 46:2821-2826.

Rowson KEK, Mahy BWJ. (1967). Human papova (wart) virus. Bacteriol Rev. 31:110-131.

Rubio CA, Lagerlof B. (1974). Studies on the histogenesis of experimentally induced cervical carcinoma. Acta Pathol Microbiol Scand. 82:153-160.

Russell JH. (1983). Internal disintegration model of cytotoxic lymphocyte-induced target damage. Immunol Rev. 72:97-118.

Rustgi AK, Dyson N, Bernards R. (1991). Amino terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. Nature. 352. 541-544.

Sadeghi SB, Hsieh EW, Gunn SW. (1984). Prevalence of cervical intraepithelial neoplasia in sexually active teenagers and young adults. Am J Obstet Gynaecol. 148:726-729.

Saiki RK, Gelfand DH, Stoffell S, et al. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239:487-491.

Saiki RK. (1989). The design and optimisation of the PCR. In: "PCR technology: principles and applications for DNA amplification." Ed. Erlich H A. Stockton press, New York, pp 7-16.

Sakata Y, Aoki N. (1982). Significance of cross-linking of alpha-2-plasmin inhibitor to fibrin in inhibition of



fibrinolysis and in haemostasis. J Clin Invest. 69:536-542.

Sanderson, CJ. (1981). The mechanism of lymphocyte-mediated cytotoxicity. Biol Rev. 56:153-197.

Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M. (1982). T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey- MSV transforming genes. Nature. 298:343-347.

Santos E, Nebreda AR. (1989). Structural and functional properties of ras proteins. FASEB J. 3:2151-2163.

Sarnow P, Ho YS, Williams J, Levine AJ. (1982). Adenovirus Elb-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. Cell. 28:387-394.

Sarraf CE, Bowen ID. (1986). Kinetic studies on a murine sarcoma and an analysis of apoptosis. Br J Cancer. 54:989-998.

Sasson IM, Haley NJ, Hoffmann D, Wynder EL, Hellberg D, Nilsson S. (1985). Cigarette smoking and neoplasia of the uterine cervix: smoke constituents in cervical mucus. N Engl J Med. 312:315.

Savill JS, Henson PM, Haslett C. (1989a). Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. J Clin Invest. 84:1518-1527.

Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. (1989b). Macrophage phagocytosis of ageing neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest. 83:865-875.

Savill JS. (1990). Macrophage recognition of senescent neutrophils. PhD Thesis. University of London.

Savill J, Dransfield I, Hogg N, Haslett C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature. 343:170-173.

Schafer WR, Kim R, Sterne R, thorner J, Kim S-H, Rine J. (1989). Genetic and pharmacological suppression of oncogenic mutations in ras genes of yeast and humans. Science. 245:379-385.

Schatten WE, Mantel N, Mider GB. (1958). A relationship between amount of necrosis in Walker carcinoma 256 and concentration of hemoglobin in the host's blood. Cancer Res. 18:274-278.

Schiffman MH, Bauer HM, Lorincz AT, et al. (1991). Comparison of southern blot hybridisation and polymerase chain reaction methods for the detection of human papillomavirus DNA. J Clin Microbiol. 29:573-577.

Schiller JT, Vass WC, Lowy DR. (1984) Identification of a second transforming region in bovine papillomavirus DNA. Proc Natl Acad Sci USA. 81:7880-7884.

Schneider A. (1990). Latent and subclinical genital HPV infections. Papillomavirus Report. 1(1):2-5.

Schneider-Gadick A, Schwar z E. (1986). Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. 5:2285-2292.

Schneider-Gadick A, Kaul S, Schwar z E, Gausepohl H, Frank R, Bastert G. (1988). Identification of the human papillomavirus type 18 E6\* and E6 proteins in nuclear protein fractions from human cervical carcinoma cells grown in the nude mouse or in vitro. Cancer Res. 48:2969-2974.

Schneider-Maunoury S, Croissant O, Orth G. (1987). Integration of human papillomavirus type 16 DNA sequences: a possible early event in the progression of genital tumours. J Virol. 61:3295-3298.

Schwab M, Ellison J, Rosenau W, Varmus HE, Bishop JM. (1984). Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc Natl Acad Sci USA. 81:4940-4944.

Schwar z E, Durst M, Demankowski C, et al. (1983). DNA sequence and organisation of genital human papillomavirus type 6b. EMBO J. 2:2341-2348.

Schwar z E, Freese U K, Gissmann L, et al. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature. 314:111-114.

Scully RE. (1976). Mitosis counting I. Hum Pathol. 7:481-482.

Searle J, Collins DJ, Harmon B, Kerr JFR. (1973). The spontaneous occurrence of apoptosis in squamous carcinomas of the uterine cervix. Pathology. 5:163-169.

Searle J, Lawson TA, Abbott PJ, Harmon B, Kerr JFR. (1975). An electron-microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J Pathol. 116:129-138.

Sebastian JA, Leeb BO, See R. (1978). Cancer of the cervix - A sexually transmitted disease. Cytologic screening in a prostitute population. Am J Obstet Gynecol. 131:620-623.

Seedorf KG, Krammer M, Durst M, Suhai S, Rowekamp WG. (1985). Human papillomavirus type 16 DNA sequence. Virology. 145:181-185.

Seedorf K, Oltersdorf T, Krammer G, Rowekamp W. (1987). Identification of early proteins of the human papillomaviruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. EMBO J. 6:139-144.

Sefton BM, Trowbridge IS, Cooper JA, Scolnick EM. (1982). The transforming protein of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell. 31:465-474.

Serra A. (1924). Studi sul virus della verruca, del papilloma, del condiloma acuminato (etiologia, patogenesi, filtrabilita); nota preventiva. Giorn Ital d Mal Venereol. 65:1808-1814.

Servomaa K, Rytomaa T. (1988). Suicidal death of rat chloroleukaemia cells by activation of the long interspersed repetitive DNA element (L1Rn). Cell Tissue Kinet. 21:33-43.

Shapiro JR, Yug W-K A, Shapiro WR. (1981). Isolation, karyotyping and clonal growth of heterogeneous populations of human malignant gliomas. Cancer Res. 41:2349-2359.

Sheffner M, Werness BA, Huibregtse JM et al. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 63:1129-1136.

Shi Y, Sahai BM, Green DR. (1989). Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. Nature. 339:625-626.

Shi Y, Bissonnette RP, Parfrey N, Szalay M, Green DR. (1991). In vivo administration of antibodies to the CD3-T cell receptor complex induces developmental cell death (apoptosis) in immature thymocytes. (manuscript submitted).

Shibata DK, Arnheim M, Martin WJ. (1988a). Detection of human papillomavirus in paraffin embedded tissues using the polymerase chain reaction. J Exp Med. 167:225-230.

Shibata D, Fu YS, Gupta JW, Shah KV, Arnheim N, Martin



JW. (1988b). Detection of human papillomavirus in normal and dysplastic tissue by the polymerase chain reaction. Lab Invest. 59:555-559.

Shimada M, Fukushima M, Mukai H, Kato I, Nishikawa A, Fujinaga K. (1990). Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. Jpn J Cancer Res. 81:1-5.

Shirasawa H, Tomita Y, Sekiya S, Takamizawa H, Simizu B. (1987). Integration and transcription of human papillomavirus type 16 and 18 sequences in cell lines derived from cervical carcinomas. J Gen Virol. 68:583-591.

Silverberg E and Lubera JA. (1989). Cancer statistics. CA - Cancer J for Clinicians. 39:3-20.

Silverberg SG. (1976). Reproducibility of the mitosis count in the histological diagnosis of smooth muscle tumours of the uterus. Hum Pathol. 7:451-454.

Skalka M, Matyasova J, Cejkova M. (1976). DNA in chromatin of irradiated lymphoid tissues degrades in vivo into regular fragments. FEBS Lett. 72: 271-274.

Slamon DJ, deKernon JB, Verma IM, Cline MJ. (1984). Expression of cellular oncogenes in human malignancies. Science. 224:256-262.

Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT. (1989). Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature. 337: 181-184.

Smith MR, DeGudicibus ST, Stacey DW. (1986). Requirement for c-ras proteins during viral oncogene transformation. Nature. 320:540-543.

Smith PP, Bryant EM, Kaur P, McDougall JK. (1989). Cytogenetic analysis of eight human papillomavirus immortalized human keratinocyte cell lines. Int J Cancer. 44:1124-1131.

Smits PHM, Smits HL, Jebbink MF, Schegget JT. (1990). The short arm of chromosome 11 likely is involved in the regulation of the human papillomavirus type 16 early enhancer-promoter and in the suppression of the transforming activity of the viral DNA. Virology. 176:158-165.

Smotkin D, Wettstein FO. (1987). The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J Virol. 61:1686-1689.

Smotkin D, Prokoph H, Wettstein FO. (1989). Oncogenic and non-oncogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J Virol. 63:1141-1147.

Snijders PJF, van den Brule AJC, Schrijnemakers HFJ, Snow G, Meijer CJLM, Walboomers JMM. (1990). The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol. 71:173-181.

Sobel ME. (1990). Metastasis suppressor genes. J Natl Cancer Inst. 82:267-276.

Southern EM. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol. 98:503-517.

Spalholz BA, Yang YC, Howley PM. (1985). Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell. 42:183-191.

Spandidos DA, Kerr IB. (1984). Elevated expression of the human ras oncogene family in premalignant and malignant tumours of the colorectum. Br J Cancer. 49:681-688.

Spandidos DA, Wilkie NM. (1984). Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature. 310:469-475.

Spandidos DA. (1985). Mechanism of carcinogenesis: the role of oncogenes, transcriptional enhancers and growth factors. Anticancer Res. 5:485-498.

Spandidos DA. (1986). A unified theory for the development of cancer. Biosci Rep. 6:691-708.

Spandidos DA. (1988). Ras oncogenes in Cell Transformation. ISI Atlas Sci.: Immunol. 1988:1-6.

Stacey NH, Bishop CJ, Halliday JW, et al. (1985). Apoptosis as the mode of cell death in antibody-dependent lymphocytotoxicity. J Cell Sci. 74:169-179.

Stanbridge EJ, Der CJ, Doersen CJ, et al. (1982). Human cell hybrids: analysis of transformation and tumorigenicity. Science. 215:252-259.

Stanbridge E. (1990). Human tumor suppressor genes. Annu Rev Genet. 24:615-657.

Stanley M. (1990). Genital papillomaviruses, polymerase chain reaction and cervical cancer. Genitourin Med. 66:415-417.

Stanton IW, Watt R, Marcu KB. (1983). Translocation, breakage and truncated transcripts of c-myc in murine plasmacytomas. *Nature*. 303:401-406.

Steel GG. (1977). Growth Kinetics of Tumours. First Edition, Clarendon Press.

Stein GH, Beeson M, Gordon L. (1990). Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. *Science*. 249:666-669.

Storey A, Pim D, Murray A, Osborn K, Banks L, Crawford L (1988). Comparison of the in vitro transforming activities of human papillomavirus types. *EMBO J*. 7:1815-1820.

Storey A, Almond N, Osborn K, Crawford L. (1990a). Mutations of the human papillomavirus type 16 E7 gene that affect transformation, transactivation and phosphorylation by the E7 protein. *J Gen Virol*. 71:965-970.

Storey A, Osborn K, Crawford L. (1990b). Co-transformation by human papillomavirus types 6 and 11. *J Gen Virol*. 71:165-171.

Sukhatme VP, Cao X, Chang S, et al. (1988). A zinc finger encoding protein co-regulated with c-fos during growth and differentiation and after depolarization. *Cell*. 53:37-43.

Syrjanen K J. (1986). Human papillomavirus (HPV) infections of the female genital tract and their associations with intraepithelial neoplasia and squamous cell carcinoma. *Path Ann*. 21:53-89.

Szende B, Zalatna A, Schally AV. (1989). Programmed cell death (apoptosis) in pancreatic cancers of hamsters after treatment with analogs of both luteinizing hormone-releasing hormone and somatostatin. *Proc Natl Acad Sci USA*. 86:1643-1647.

Tamanai F. (1988). Yeast ras genes. *Biochim Biophys Acta*. 948:1-15.

Tannock IF. (1968). The relationship between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br J Cancer*. 22:258-273.

Tase T, Okagaki T, Clark BA, et al. (1988). Human papillomavirus types and localization in adenocarcinoma and adenosquamous carcinoma of the uterine cervix: a study by in situ DNA hybridisation. *Cancer Res*. 48:993-998.

Taub R, Kirsch I, Morton C, et al. (1982). Translocation

of the c-myc gene into the immunoglobulin heavy gene locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA. 79:7837-7841.

Teyssier JR. (1989). The chromosomal analysis of human solid tumors: a triple challenge. Cancer Genet Cytogenet. 37:103-125.

Thomlinson RH, Gray LH. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer. 9:539-549.

Thompson CB, Challoner PB, Neiman PE, Grondine M. (1985). Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. Nature. 314:363-365.

Thorgeirson VP, Turpeenniemi-Hujanen T, Williams JE, et al. (1985). NIH/3T3 cells transfected with human tumour DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. Mol Cell Biol. 5:259-262.

Tidy JA, Farrell PJ. (1989). Retraction: human papillomavirus subtype 16b. Lancet II:1535.

Tidy JA, Parry GCN, Ward P, Coleman DV, Peto J, Malcolm ADB, Farrell PJ. (1989a). High rate of HPV 16 infection in the female population. Lancet. I:434.

Tidy JA, Vousden KH, Farrell PJ. (1989b). Relation between infection with a subtype of HPV 16 and cervical neoplasia. Lancet.I:1225-1227.

Tidy J, Vousden KH, Mason P, Farrell PJ. (1989c). A novel deletion within the upstream regulatory region of episomal human papillomavirus type 16. J Gen Virol. 70:999-1004.

Toon PG, Arrand JR, Wilson LP, Sharp DS. (1986). Human papillomavirus infection of the uterine cervix of women without cytological signs of neoplasia. Br Med J. 293:1261-1264.

Tozer GM, Lewis S, Michalowsky A, Aber V. (1990). The relationship between regional variations in blood flow and histology in a transplanted rat fibrosarcoma. Br J Cancer. 61:250-257.

Trauth BC, Klas C, Peters AMJ, et al. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science. 245:301-305.

Tremethick DJ, Molloy PL. (1988). Effects of high mobility group proteins 1 and 2 on initiation and elongation of specific transcription by RNA polymerase II in vitro. Nucl Acids Res. 16:11107-11123.

Trump BF, Berezesky IK, Osornio-Vargas AR. (1981). Cell death and the disease process. The role of calcium. In "Cell Death in Biology and Pathology". Eds. Bowen ID, Lockshin RA. Chapman and Hall, London. 209-242.

Udvardy A, Schedl P, Sander M, Hsieh TS. (1986). Topoisomerase II cleavage in chromatin. J Mol Biol. 191:231-246.

Umansky SR, Korol BA, Nelipovich PA. (1981). In vivo DNA degradation in thymocytes of x-irradiated or hydrocortisone-treated rats. Biochim Biophys Acta. 655:9-17.

Van Beneden RJ, Watson DK, Chen TT, Lautenberger JA, Papas TS. (1986). Cellular myc (c-myc) in fish (rainbow trout): Its relationship to other vertebrate myc genes and to the transforming genes of the MC29 family of viruses. Proc Natl Acad Sci USA. 83:3698-3702.

van Bommel PFJ, Lindert ACM, Kock HCLV, Leers WH, Neijt JP. (1987). A review of prognostic factors in early-stage carcinoma of the cervix (FIGO IB and IIA) and implications for treatment strategy. Eur J Obstet Repro Biol. 26:69-84.

van den Brule AJC, Claas ECJ, Du Maine M, et al. (1989). Use of anticontamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. J Med Virol. 29:20-27.

Van Haelst U. (1967a). Light and electron microscopic study of the normal and pathological thymus of the rat. Z. Zellforsch Mikrosk Anat. 77:534-553.

Van Haelst U. (1967b). Light and electron microscopic study of the normal and pathological thymus of the rat. II. The acute thymic involution. Z. Zellforsch Mikrosk Anat. 80:153-182.

Van Nagell JR, Rayburn W, Donaldson ES et al. (1979). Therapeutic complications of patterns of recurrence in cancer of the uterine cervix. Cancer. 44:2354-2361.

Varmus HE. (1984). The molecular genetics of cellular oncogenes. Annu Rev Genet. 18:553-612.

Vaux DL, Cory S, Adams JM. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 335:440-442.

Vessey MP. (1986). Epidemiology of cervical cancer: role of hormonal factors, cigarette smoking and occupation. In: Origins of female genital cancer: virological and



epidemiological aspects. Eds: Peto R, zur Hausen H. Banbury Report, Cold Spring Harbor Lab Press, N Y. 29-43.

Vindelov LL, Christensen IJ, Nissen N. (1983a). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry*. 3:323-327.

Vindelov LL, Christensen IJ, Nissen N. (1983b). Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. *Cytometry*. 3:328-331.

Vogt M, Lesley J, bogenberger J, Volkman S, Haas M. (1986). Coinfection with virus carrying the v-Ha-ras and v-myc oncogenes leads to growth factor independence by an indirect mechanism. *Mol Cell Biol*. 6:3545-3549.

Vonka V, Kanka J, Hirsch I, et al. (1984). Prospective study on the relationship between cervical neoplasia and herpes simplex type-2 virus. II Herpes simplex type-2 antibody presence in sera taken at enrolment. *Int J Cancer*. 33:61-66.

Vonka V, Kanka J, Roth Z. (1987). Herpes simplex type II and cervical neoplasia. *Adv Cancer Res*. 48:149-191.

Vousden KH, Marshall CJ. (1984). Three different activated ras genes in mouse tumours; evidence for oncogene activation during progression of a mouse lymphoma. *EMBO J*. 3:913-917.

Vousden KH, Eccles SA, Purvies H, Marshall CJ. (1986). Enhanced spontaneous metastasis of mouse carcinoma cells transfected with an activated c-Ha-ras-1 gene. *Int J Cancer*. 37:425-433.

Vousden KH, Doniger J, DiPaolo JA, Lowy DR. (1988). The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene Res*. 3:167-175.

Vousden KH, Androphy EJ, Schiller JT, Lowy DR. (1989). Mutational analysis of bovine papillomavirus E6 gene. *J Virol*. 63:2340-2342.

Vousden KH, Jat PS. (1989). Functional similarity between HPV 16 E7, SV40 large T and adenovirus E1a proteins. *Oncogene*. 4:153-158.

Waelsh L. (1918). Uebertragungsversuche mit spitzem kondylom. *Arch f Dermatol u Syph*. 124:625-626.

Wagatsuma M, hashimoto K, Matsukura T. (1990). Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. *J Virol*.

64:813-821.

Wagner S, Green MR. (1991). Retinoblastoma: a transcriptional tryst. *Nature*. 352:189-190.

Walker NI. (1987). Ultrastructure of the rat pancreas following experimental duct ligation. I. The role of apoptosis and intraepithelial macrophages in acinar duct deletion. *Am J Pathol*. 126:439-451.

Walker NI, Gobe GC. (1987). Cell death and cell proliferation during atrophy of the rat parotid gland induced by duct obstruction. *J Pathol*. 153:333-344.

Walker NI, Bennett RE, Axelson RA. (1988a). Melanosis coli-a consequence of anthroquinane-induced apoptosis of colonic epithelial cells. *Am J Pathol*. 131:465-476.

Walker NI, Harmon BV, Gobe GC, Kerr JFR. (1988b). Patterns of cell ceath. *Meth Archiev Exp Path*. 13:18-54.

Walker NI, Bennett RE, Kerr JFR. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am J Anat*. 185:19-32.

Ward P, Coleman DV, Malcolm DB. (1989). Regulatory mechanisms of the papillomaviruses. *Trends in Genetics*. 5:97-99.

Waring P, Eichner RD, Mullbacher A, Sjaarda A. (1988). Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. *J Biol Chem*. 263:18493-18499.

Watanabe S, Kanda T, Yoshiike K. (1989). Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *J Virol*. 63:965-969.

Watanabe S, Kanda T, Sato H, Furuno A, Yoshiike K. (1990). Mutational analysis of human papillomavirus type 16 E7 functions. *J Virol*. 64:207-214.

Waters CM, Hancock DC, Evan GI, et al. (1990). Identification and characterization of the egr-1 gene product as an inducible short-lived nuclear phosphoprotein. *Oncogene* 5:669-674.

Waters CM, Littlewood D, Hancock DC, et al. (1991). C-myc protein expression in untransformed fibroblasts. *Oncogene* 6:797-805.

Watson DK, Psallidopoulos MC, Samuel KP, Dalla-Favera R, Papas TS. (1983). Nucleotide sequence analysis of human c-myc locus, chicken homologue and myelocytomatosis virus

- MC29 transforming gene reveals a highly conserved gene product. Proc Natl Acad Sci USA. 80:3642-3645.
- Watts SL, Phelps WC, Ostrow RS, Zachow KR, Faras AJ. (1984). Cellular transformation by human papillomavirus DNA in vitro. Science. 225:634-636.
- Weid GL. (1961). Proceedings of the First International Congress on Exfoliative Cytology. 1st ed. Philadelphia, J B Lippincott.
- Weinberg RA. (1991). Tumor suppressor genes. Science 254:1138-1146.
- Weiss RJ, Lucas WE. (1986). Adenocarcinoma of the uterine cervix. Cancer. 57:1996-2001.
- Werness BA, Levine AJ, Howley PM. (1990). Association of human papillomavirus types 16 and 18 proteins with p53. Science. 248:76-79.
- WHO Meeting. (1986). Control of cancer of the cervix uteri: Bull WHO 64:607-618.
- Whyte P, Buchkovich KJ, Horowitz JM et al. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature. 334:124-129.
- Wilcock C, Chahwala SB, Hickman JA. (1988). Selective inhibition by bis (2-chloroethyl) methylamine (nitrogen mustard) of the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransporter of murine L1210 leukaemia cells. Biochim Biophys Acta. 946:368-378.
- Wilczynski S P, Bergen S, Walker J, Liao S-Y, Pearlman L F. (1988). Human papillomaviruses and cervical cancer: analysis of histopathologic features associated with different viral types. Hum Pathol. 19:697-704.
- Williams ARW, Piris J, Spandidos DA, Wyllie AH. (1985). Immunohistochemical detection of the ras oncogene p21 product in an experimental tumour and in human colorectal neoplasms. Br J Cancer. 52:687-693.
- Williams ARW, Piris J, Wyllie AH. (1990). Immunohistochemical demonstration of altered intracellular localization of the c-myc oncogene product in human colorectal neoplasms. J Pathol. 160:287-293.
- Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR. (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature. 343:76-79.
- Williams GT. (1991). Programmed cell death: apoptosis and oncogenesis. Cell. 65:1097-1098.



Winkler B, Capo V, Reumann W, et al. (1985). Human papillomavirus infection of the oesophagus. A clinicopathologic study with demonstration of papillomavirus antigen by the immunoperoxidase technique. *Cancer*. 55:149-155.

Winqvist R, Saksela K, Alitalo K. (1984). The myc proteins are not associated with chromatin in mitotic cells. *EMBO J*. 3:2947-2950.

Woodworth CD, Bowden PE, Doniger J, et al. (1988). Characterization of normal human exocervical epithelial cells immortalized in vitro by papillomavirus types 16 and 18 DNA. *Cancer Res*. 48:4620-4628.

Woodworth CD, Doniger J, DiPaolo JA. (1989). Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. *J Virol*. 63:159-164.

Wyllie AH, Kerr JFR, Currie AR. (1973a). Cell death in the normal neonatal rat adrenal cortex. *J Pathol*. 111:255-261.

Wyllie AH, Kerr JFR, Macaskill IAM, Currie AR. (1973b). Adrenocortical cell deletion: the role of ACTH. *J Pathol*. 111:85-94.

Wyllie AH. (1980). Glucocorticoid induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*. 284:555-556.

Wyllie AH, Kerr JFR, and Currie AR. (1980). Cell Death: The significance of apoptosis. *Int Rev Cytol*. 68:251-306.

Wyllie AH. (1981). Cell death: a new classification separating apoptosis from necrosis. In "Cell Death in Biology and Pathology". Eds. Bowen ID, Lockshin RA. Chapman and Hall Press, London. 9-34.

Wyllie AH, Morris RG. (1982). Hormone-induced cell death, purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am J Pathol*. 109:78-87.

Wyllie AH, Duvall E, Blow JJ. (1984a). Intracellular mechanisms in cell death in normal and pathological tissues. In "Cell ageing and cell death". Eds. Davis I, Sigeo DC. Cambridge University Press. pp.269-294.

Wyllie AH, Morris RG, Smith AL, Dunlop D. (1984b). Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol*. 142:67-77.

Wyllie AH. (1985). The biology of cell death in tumours. *Anticancer Res.* 5:131-136.

Wyllie AH, Morris RG, Arends MJ, Watt AE. (1986a). Nuclease activation in programmed cell death. In: "Coordinated regulation of gene expression". Eds Clayton RM, Truman DES. Plenum Press, New York. 33-41.

Wyllie AH, Morris RG, Watt AE. (1986b). Terminally differentiated and dying lymphoid cells contain nuclear endonuclease potentially responsible for chromatin changes of apoptosis. *J Pathol.* 148:94A.

Wyllie AH. (1987a). Cell death. *Int Rev Cytol (Suppl).* 17:755-785.

Wyllie AH. (1987b). Apoptosis: cell death under homeostatic control. *Arch Toxicol (Suppl).* 11:3-10.

Wyllie AH. (1987c). Oncogenes: correlates or controls of tumour progression. In: "Causation and prevention of colorectal cancer". Eds. Faivre J, Hill MJ. Elsevier Science Publishers BV. pp121-132.

Wyllie AH, Rose KA, Morris RG, Steel CM, Foster E, Spandidos DA. (1987). Rodent fibroblast tumours expressing human myc and ras genes: growth, metastasis and endogenous oncogene expression. *Br J Cancer.* 56:251-259.

Wyllie AH. (1988). Apoptosis. *ISI Atlas Science: Immunology.* 1: 192-196.

Wyllie AH, Evans I, Morris RG, Spandidos DA. (1989). Regulation of cell death by ras and myc oncogenes. In "Ras oncogenes". Ed. Spandidos DA. Plenum Press. New York. 149-155.

Xiao X, Cao M, Miller TR, Cao Z-Y, Yen BTS. (1988). Papillomavirus DNA in cervical carcinoma specimens from central China. *Lancet.* II:902.

Yajima A, Fukuda M, Noda K. (1984). Histopathological findings concerning the morphogenesis of mixed carcinoma of the uterine cervix. *Gynecol Oncol.* 18:157-164.

Yamada T, and Ohyama H. (1980). Changes in surface morphology of rat thymocytes accompanying interphase death. *J Rad Res.* 21: 190-196.

Yamada T, Ohyama H. (1988). Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). *Int J Radiat Biol.* 53:65-75.

Yang Y-C, Spalholz B A, Rabson M S, Howley P M. (1985).

Dissociation of transforming and transactivation functions for bovine papillomavirus type 1. *Nature*. 318:575-577.

Yasumoto S, Burkhardt A L, Doniger J, DiPaolo D A. (1986). Human papillomavirus type 16 DNA-induced malignant transformation of NIH3T3 cells. *J Virol*. 57:572-577.

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. (1991). Wild type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*. 353:345-347.

Young FI, Ward LM, Brown LJR. (1991). Absence of human papillomavirus in cervical adenocarcinoma determined by in-situ hybridisation. *J Clin Pathol*. 44:340-341.

Young LS, Bevan IS, Johnson MA, et al. (1989). The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. *Br Med J*. 298:14-18.

Yuan J, Horvitz HR. (1990). The *Caenorhabditis* genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Devel Biol*. 138:33-41.

Yutsudo M, Okamoto Y, Hakura A. (1988). Functional dissociation of transforming genes of human papillomavirus type 16. *Virology*. 166:594-597.

Zahn X, Goldfarb M. (1986). Growth factor requirements of oncogene-transfected NIH3T3 and BALB/c 3T3 cells cultured in defined media. *Mol Cell Biol*. 6:3541-3544.

Zajicek G, Oren R, Weinreb M. (1985). The streaming liver. *Liver*. 5:293-300.

Zelevnik AJ, Ihrig LL, Bassett SG. (1989). Developmental expression of Ca<sup>++</sup>/Mg<sup>++</sup>-dependent endonuclease activity in rat granulosa and luteal cells. *Endocrinology*. 125:2218-2220.

Zhang K, DeClue DE, Vass WC, Papageorge AG, McCormick F, Lowy DR. Suppression of c-ras transformation by GTPase-activating protein. *Nature* 346:754-756.

zur Hausen H. (1977). Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol*. 78:1-30.

zur Hausen H. (1980). The role of viruses in human tumours. *Adv Cancer Res*. 33:77-107.

zur Hausen H. (1982). Human genital cancer: synergism between two virus infections or synergism between a virus

infection and initiating events. Lancet. II:1370-1372.

zur Hausen H. (1986). Intracellular surveillance of persisting viral infections: human genital cancer results from deficient cellular control of papillomavirus gene expression. Lancet. II:489-491.

zur Hausen H. (1989). Papillomaviruses as carcinomaviruses. In: Advances in viral oncology. Ed. Klein G. New York: Raven Press, pp 1-26.

Blackwood EM, Eisenman RN. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. Science. 251:1211-1217.